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(54) Title: METHODS AND REAGENTS FOR THE DETERMINATION OF IMMUNOSUPPRESSIVE AGENTS

(57) Abstract

Assay methods and reagents for determining the presence or amount of immunophilin ligands and immunophilins thereof employing a recombinant fusion protein comprising (i) an immunosuppressant binding protein and (ii) a heterologous protein are disclosed. The recombinant fusion protein can also be employed for the evaluation of immunosuppressive activities of immunosuppressive agents in order to determine the efficacy of an immunosuppressive agent during the course of therapeutic treatment of a patient therewith. Preferably, the recombinant fusion protein comprises a macrolide immunosuppressive agent and CTP:CMP-3-deoxy-D-*manno*-octulosonate cytidylyl transferase. When employed in a binding assay format, the recombinant fusion protein provides higher reactivity for the immunophilin ligand under determination than does the native immunosuppressant binding protein. In particular, an immunosuppressant assay reagent comprising FK-506 binding protein (FKBP) and CKS immobilized to a solid support material provides a higher signal-to-noise ratio when employed in a competitive heterogeneous assay format than when native FKBP immobilized to a solid support material is employed in such assay format.

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# METHODS AND REAGENTS FOR THE DETERMINATION OF IMMUNOSUPPRESSIVE AGENTS

## Field of the Invention

The present invention relates to the determination of immunosuppressive agents in a test sample. In particular, the present invention relates to quantitative assays for immunosuppressive agents, and immunophilins of such immunosuppressive agents, employing recombinant fusion proteins comprising a specific binding protein for an immunosuppressive agent and a heterologous protein.

## Background of the Invention

Immunosuppressive agents such as FK-506, cyclosporin and rapamycin are macrocyclic drugs of *Streptomycete* origin having *in vivo* and *in vitro* immunosuppressive properties [A. W. Thomson, Immunol. Today, Volume 10, pages 6-9 (1989), and B. D. Kahan, et. al., Transp. , Volume 52, pages 185-191 (1991)]. Such immunosuppressive agents appear to selectively act on T lymphocytes and inhibit the production of, or responses to, T lymphocyte growth and differentiation enhancing lymphokines such as interleukin-2 (IL-2 ) [F. J. Dumont, et. al., J. Immunol., Volume 144, pages 51-2581(1990)], and are therefor useful for the treatment of transplantation rejection and autoimmune diseases. For example, European Patent Application Publication Number 184,162 describes the macrolide immunosuppressant FK-506 which is effective in the dose range of between 0.2 ng/mL and 2.0 ng/ml in plasma [W. J. Jusko, et al., Transp. Proc., Volume 23, No. 6, pages 2732-2735 (1991)] and 15 to 20 ng/mL in whole blood [Japanese FK-506 Study Group, Transp. Proc., Volume 23, No. 6, pages 3071-3074 (1991).

Accordingly, the monitoring of therapeutic drug levels of such immunosuppressive agents in biological fluids has become very useful to provide physicians with information to aid in patient management. The monitoring of such

immunosuppressive agent levels enables adjustment of patient dosage to achieve optimal therapeutic effects, and helps avoid either subtherapeutic or toxic levels. For example, *in vivo* evaluations of FK-506 have demonstrated toxic effects [J. J. Fung, et al., Transp. Proc., Volume 23, pages 3105-3108 (1991) and T. Ochiai, et al., Transp. Proc., Volume 23, pages 2718-2721 (1991)]. Moreover, initial trials with FK-506 suggest that careful maintenance of whole blood levels can minimize the toxic effects while maximizing immunosuppressive efficacy [Japanese FK-506 Study Group, Transp. Proc., Volume 23, pages 3071-3074 and 3085-3088 (1991)]. However, the need still exists to provide assay methods which are sensitive to detect low blood levels of immunosuppressive agents without detecting interfering non-immunosuppressive metabolites thereof.

Macrolide immunosuppressive agents have been quantitated in solution or bodily fluids at concentrations of 50 ng/ml or higher using high performance liquid chromatography and detection by ultraviolet absorbance [M. C. Friob, et al., Transp. Proc., Volume 23, pages 2750-2752 (1991) and K. L. Napoli, et. al., Clin. Chem., Volume 37, page 275 (1991)]. However, clinically effective doses of FK-506 result in trough whole blood levels of 15-20 ng/ml [Japanese FK-506 Study Group, Transp. Proc., Volume 23, pages 3071-3074 and 3085-3088 (1991)] which are difficult to detect. Although clinically effective doses of rapamycin have not yet been determined, it has been proposed that sensitivity of detection below 1 ng/ml will be necessary to monitor therapeutically effective doses of the molecule [B. D. Kahan, et. al., Transp., Volume 52, pages 185-191 (1991)]. For example, the determination of therapeutic drug levels of FK-506 can be accomplished in an immunoassay employing anti-FK-506 antibody and drug-enzyme conjugates [Tamura, et. al., Transp. Proc., Volume 19, pages 23-29 (1987) and European Patent Application Publication Number 293892)]. The reported minimum concentration of FK-506 detected in such ELISA assay format has been 0.1 ng/ml in plasma and 0.7 ng/ml in whole blood [W. J. Jusko and R. D'Ambrosio, Transp. Proc., Volume 23, pages 2732-2735 (1991)].

Tissues which are the target for macrolide immunosuppressive agents such as FK506 and rapamycin contain protein receptors, known as immunophilins or immunosuppressant binding proteins, which specifically bind to their respective immunosuppressive agent [J. Siekierka, et. al., Nature, Volume 341, pages 755-757 (1989) and M. Harding, et. al., Nature, Volume 341, pages 758-760 (1989)], and assays employing such protein receptors for detecting macrolide immunosuppressive agents and their metabolites have also been described. For example, European Patent Publication Number 379,342 and International Patent

Publication No. WO91/04321 describe a cytosolic binding protein, FK-506 binding protein (FKBP), which binds FK-506 and structurally related drugs. FKBP exists in a soluble form from cellular sources [J. Siekierka, et al., J. Immunol., Volume 143, pages 1580-1583 (1989)] and can be produced by recombinant techniques [R. Standaert, et al., Nature, Volume 346, pages 671-674 (1990)]. In addition, such binding protein can be used to determine the presence or quantity of biologically useful ligand (European Patent Publication Number 379,342), or may be used to purify material binding to FKBP-ligand complexes such as a calcium/calmodulin phosphatase whose interaction with the FKBP-ligand complex may be especially relevant to the immunosuppressive actions of FK-506 [J. Liu, et al., Cell, Volume 66, pages 807-815 (August 1991)], whereby a glutathione S-transferase-FKBP fusion protein was used in place of FKBP.

Although the crossreactivity of all metabolites of FK506 in an immunoassay does not correspond to the immunosuppressive activity [N. Kobayashi, et al., Transp. Proc., Volume 23, pages 2725-2729 (1991)], use of an immunophilin-based assay may provide a better correlation between assay crossreaction and immunosuppressive potential of drug metabolites [Lorber, et al., Transp. Proc., Volume 22, pages 1240-1244 (1990)]. For example, a binding protein based assay for cyclosporin was found to show binding of cyclosporin analogs in proportion to their immunosuppressive activity [R. Handschumacher, et al., Science, Volume 226, pages 544-546 (1984). A variation of this assay in a side by side comparison with an HPLC method has also been described [Lorber, et al., Transplant Proc., Volume 22, No. 3, pages 1240-1244 (1990)].

In the case of immunoassays, FKBP, like other proteins, can be affixed to a solid phase material by passive adsorption (A. Voller, et al., In Manual of Clinical Immunology (eds. Rose N. & Feldman H), page 506 (1976), American Society for Microbiology, Washington, D.C.). However, as is well known in the art, the immobilization of proteins to solid phase materials, such as by adsorption or covalent binding, may cause conformational changes in the protein, leading to loss of activity. In addition, the immobilization process may be subject to variation due to poor control of pH, time and temperature.

## Summary of the Invention

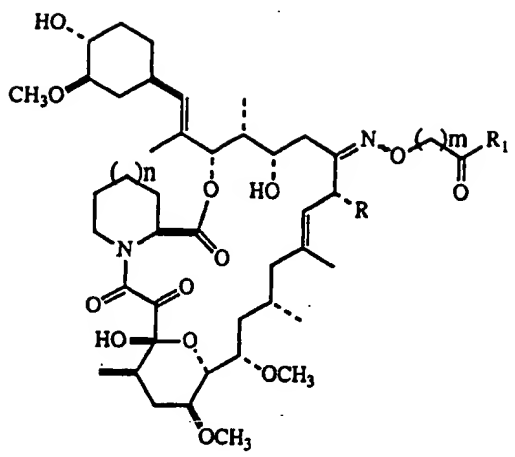
The present invention provides assay methods and reagents for evaluating immunophilin ligands such as immunosuppressive agents and other agents capable of binding to immunosuppressant binding proteins, and immunophilins thereof, in

a test sample from a patient undergoing therapeutic treatment therewith. Such evaluation is accomplished employing an immunosuppressant assay reagent according to the present invention in a binding assay format whereby the amount or presence of such immunophilin ligands or immunophilins can be determined or, alternatively, the immunosuppressive activities of immunosuppressive agents and immunosuppressive metabolites can be evaluated in order to determine the efficacy of an administered immunosuppressive agent during the course of therapeutic treatment of a patient therewith.

The immunosuppressant assay reagent of the present invention is a recombinant fusion protein comprising (i) an immunosuppressant binding protein and (ii) a heterologous protein. The immunosuppressant binding protein of the immunosuppressant assay reagent, as contemplated by the present invention, is a protein which is capable of binding to an immunophilin ligand. The heterologous protein of the immunosuppressant assay reagent, as contemplated by the present invention, is a protein which is capable of being fused to the immunosuppressant binding protein, preferably CTP:CMP-3-deoxy-D-*manno*-octulosonate cytidylyl transferase, also known in the art as CMP-KDO synthetase or CKS, an enzyme derived from *Escherichia coli* (*E. coli*), according to methods know in the art,

When employed in a specific binding assay format, the immunosuppressant assay reagent of the present invention provides higher reactivity with the immunophilin ligand under determination than does the native immunosuppressant binding protein. Moreover, the present inventors have surprisingly and unexpectedly found that the heterologous protein component renders the immunosuppressant assay reagent more amenable than the native immunosuppressant binding protein to binding immunophilin ligands when immobilized to a solid support material. In particular, an immunosuppressant assay reagent comprising FK-506 binding protein (FKBP) and CKS immobilized to a solid support material provides a higher signal-to-noise ratio when employed in a competitive heterogeneous assay format than when native FKBP immobilized to a solid support material is employed in such assay format.

The present invention also provides compounds of the formulae:

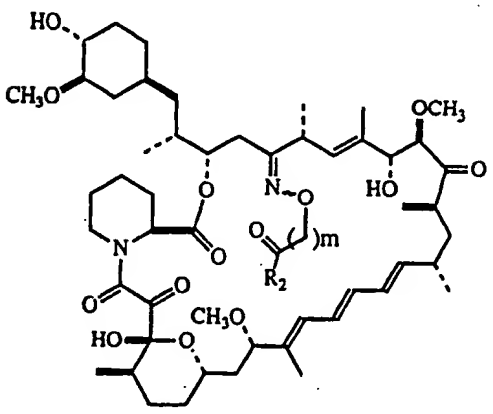


wherein n is the integer 0 or 1;

m is an integer from 0-6;

R is selected from the group consisting of methyl, ethyl, propyl and allyl; and

$R_1$ is OH or NH-X, wherein NH-X is a macromolecule or a detectable moiety, and



wherein m is an integer from 0-6; and

$R_2$ is OH or NH-Y, wherein NH-Y is a macromolecule or a detectable moiety, all of which are useful for preparing reagents employed according to the methods of the present invention.

## Brief Description of the Drawings

Figure 1 is a graphic representation of a DNA cloning vehicle for preparing the immunosuppressant assay reagent of the present invention.

Figure 2 compares the binding of an ascomycin-alkaline phosphatase detectable reagent of the present invention to immobilized forms of FKBP and the FKBP-CKS immunosuppressant assay reagent of the present invention as described in Example 9.

Figure 3 illustrates the inhibition of binding of an ascomycin-alkaline phosphatase detectable reagent to an immobilized form of the FKBP-CKS immunosuppressant assay reagent of the present invention by FK-506 and rapamycin as described in Example 9.

Figure 4 illustrates the total and nonspecific binding of a [$^3$H]-dihydro-FK-506 detectable reagent to an immobilized form of the FKBP-CKS immunosuppressant assay reagent of the present invention as described in Example 10.

Figure 5 illustrates the total and nonspecific binding signal when a multicomponent ligand system is used as a detectable reagent for binding to an immobilized form of the FKBP-CKS immunosuppressant assay reagent of the present invention as described in Example 11.

Figure 6 illustrates the inhibition of a multicomponent ligand system from binding to an immobilized form of the FKBP-CKS immunosuppressant assay reagent of the present invention by FK-506, rapamycin, and ascomycin as described in Example 11.

Figures 7A and 7B illustrate the detection of ascomycin from mouse whole blood and plasma employing an immobilized form of the FKBP-CKS immunosuppressant assay reagent of the present invention as described in Example 12.

Figure 8 illustrates the binding of calcineurin to an immobilized form of the FKBP-CKS immunosuppressant assay reagent as described in Example 13.

Figure 9 illustrates the dependence of calcineurin binding to an immobilized form of the FKBP-CKS immunosuppressant assay reagent of the present invention on the concentration of FK-506 and ascomycin as described in Example 13.

Figure 10 illustrates the use of an immobilized form of the FKBP-CKS immunosuppressant assay reagent in an assay for FKBP binding activity as described in Example 14.

Figure 11 illustrates the use of an immobilized form of the FKBP-CKS immunosuppressant assay reagent of the present invention in an assay for FK-506 wherein the immunosuppressant assay reagent is immobilized to a solid phase material with polyclonal or monoclonal anti-CKS antibodies as described in Example 15a.

Figure 12 illustrates the ability of polyclonal and monoclonal anti-CKS antibodies to separate free ascomycin-alkaline phosphatase detectable reagent from detectable reagent bound to the FKBP-CKS immunosuppressant assay reagent of the present invention as described in Example 15b.

Figure 13 illustrates the synthetic pathway for preparing reagents of the present invention derived from ascomycin and ascomycin-analogs.

Figure 14 illustrates the synthetic pathway for preparing reagents of the present invention derived from rapamycin and rapamycin-analogs.

## Detailed Description of the Invention

### *Immunophilin Ligands*

Immunophilin ligands which can be determined and evaluated according to the present invention include, but are not intended to be limited to, macrolide immunosuppressive agents, other agents capable of binding to immunosuppressant binding proteins, and the like. In particular, such immunophilin ligands contemplated by the present invention include, but are not intended to be limited to, FK-506, rapamycin, cyclosporin, ascomycin, and analogs and synthetic derivatives thereof, and the like. Other immunophilin ligands contemplated by the present invention include, but are not intended to be limited to, non-macrocyclic compounds and non-immunosuppressive agents which are capable of interacting with immunosuppressant binding proteins, such as those described in International Patent Application Publication No. WO/92-04370, heat-shock proteins and glucocorticoid receptors [(P.-K.K. Tai, et al., Science, Volume 256, pages 1315-1318 (1992)], and the like.

As used herein, immunophilin ligands also include their respective biologically-active metabolites, derivatives, and analogs thereof. Immunophilin ligand-analogs include those substances which cross-react with an immunosuppressant binding protein for the immunophilin ligand of interest, although it may do so to a greater or lesser extent than does the immunophilin ligand itself. The immunophilin ligand-analog can include a modified immunophilin ligand-analog as well as a fragmented or synthetic portion of the immunophilin ligand molecule, provided that the immunophilin ligand-analog has at least one epitopic site in common with the immunophilin ligand of interest. For example, an immunophilin ligand-analog can be a synthetic peptide sequence which duplicates or mimics at least one epitope of the whole immunophilin ligand

molecule so that the immunophilin ligand-analog can bind to an immunophilin. Other immunophilin ligands which can be determined and evaluated according to the present invention include those immunophilin ligands obtained from fermentation, chemical synthesis, and the like.

*Immunosuppressant Assay Reagent*

The immunosuppressant assay reagent of the present invention is a recombinant fusion protein comprising an immunosuppressant binding protein and a heterologous protein, wherein the immunosuppressant binding protein can be fused to a heterologous protein according to methods known in the art. Immunosuppressant binding proteins contemplated by the present invention are specific binding proteins capable of binding to the immunosuppressive agent under determination and include, but are not intended to be limited to FK-506-binding protein, rapamycin-binding protein, cyclosporin-binding protein, and the like. For example, although the predominant FKBP found in T-cells has been identified as FKBP-12, other immunosuppressant binding proteins which have been derived from multigene families, such as FKBP-13, FKBP-25, and cyclophilin, are contemplated by the present invention.

It is to be understood that the characteristics of the immunosuppressant binding proteins are such that they may crossreact with some immunophilin ligands, wherein the specificity of the various assay methods and immunosuppressant assay reagent of the present invention will be mirrored by the specificity of the interaction between the immunosuppressant binding protein and the immunophilin ligand. For example, in a binding assay employing a FKBP-CKS immunosuppressant assay reagent of the present invention as described herein, either FK-506, ascomycin, or rapamycin, would be detected, whereas cyclosporin A would not. On the other hand, in a binding assay employing a cyclosporin binding protein (cyclophilin)-CKS immunosuppressant assay reagent of the present invention, cyclosporins would be detected whereas FK-506, ascomycin or rapamycin would not.

The heterologous protein is a protein, other than the immunosuppressant binding protein, which preferably has little or no specific binding reactivity with the immunophilin ligand of interest. In addition, the heterologous protein preferably provides sufficient bulk or mass to allow immobilization of the immunosuppressant assay reagent to a solid phase material as described herein while, at the same time, retaining binding activity of the immunosuppressant assay

reagent for immunophilin ligands. Such heterologous proteins include, but are not intended to be limited to, CKS; glutathione S-transferase; the Fc portion of immunoglobulin molecules; and the like.

As would be understood by one skilled in the art, prokaryotic or eukaryotic proteins can be expressed in hosts where such proteins are not normally present, i.e., proteins which are heterologous to the host. Generally, such protein expression is accomplished in a recombinant fusion system by inserting the deoxyribonucleic acid (DNA) sequence which codes for the protein of interest downstream from a control region (e.g., a *lac* operon) in plasmid DNA, which plasmid is inserted into the cell to transform the cell so it can produce (or express) the fusion protein of interest (Figure 1). The carrier portion of a hybrid gene, typically found on the 5' end of the gene, provides the regulatory regions for transcription and translation as well as providing the genetic code for a peptide which facilitates detection [Shuman, et al., J. Biol. Chem., Volume 255, page 168 (1980)] and purification [(Moks, et al., Bio/Technology, Volume 5, page 379 (1987)]. Frequently, potential proteolytic cleavage sites are engineered into the fusion protein to allow for the removal of the homologous peptide portion [De Geus, et al., Nucleic Acid Res., Volume 15, page 3743 (1987); Nambiar, et al., Eur. J. Biochem., Volume 163, page 67 (1987); and Imai, et al., J. Biochem., Volume 100, page 425 (1986)].

Preferably, the immunosuppressant assay reagent is a recombinant fusion protein comprising an immunosuppressant binding protein and CKS. Such fusion protein can be prepared according to the methods described in copending U.S. Patent Application Serial No. 276,263, filed November 23, entitled "CKS Method Of Protein Synthesis and in Biotechniques, Volume 8, pages 488-490 (1990), both of which are incorporated herein by reference. According to such method, a fusion protein of CKS and a heterologous protein is expressed in cells transformed with a cloning vehicle which has a DNA insert coding for CKS and the heterologous protein. Such DNA cloning vehicle includes a control region, a region coding at least a portion of CKS, and a region coding for the protein of interest. The control region includes a modified *lac* promoter which is essentially native *lacP* from -73 to +21 with a deletion at -24 of one Guanine/Cytosine nucleic acid base pair and a deletion at the -9 position, and directs expression of the coding regions. The control region also includes a synthetic ribosome binding site (nt 31-39) which is homologous to the 3' end of the 16S ribosomal ribonucleic acid (rRNA) in *E. Coli*. Following the ribosome binding site is a consensus spacer region which is followed by the ATG translational codon, followed by the structural gene for

CKS. The sequence for the structural gene encoding CKS (the *kdsb* gene), and the amino acid sequence for CKS derived from the DNA sequence, are described in Goldman, et al., J. Biol. Chem., Volume 261, page 15831 (1986). Generally, the method for expressing a protein in such fusion system comprises the steps of (i) providing a DNA cloning vehicle as described above, (ii) transforming a microbe with such DNA cloning vehicle, and (iii) expressing the fusion protein comprising the protein of interest and CKS.

According to a preferred embodiment of the present invention, the immunosuppressant assay reagent comprises a recombinant FKBP-CKS fusion protein. Such fusion protein can be prepared by isolating the human FKBP gene from a Jurkat T cell cDNA library and incorporating it into an *E. coli* expression vector containing the CKS gene under the control of a *lac* promoter. The DNA for FKBP-CKS fusion protein was prepared from the FKBP gene by cloning into an expression vector containing the CKS gene under the control of a modified *lac* promoter as described by T. Bolling and W. Mandecki, Biotechniques, Volume 8, pages 488-490 (1990). The fusion protein preparation was obtained from lysed *E. coli* by 25 to 35% ammonium sulfate fractionation. Cytosols from cells expressing the FKBP-CKS fusion protein or purified FKBP-CKS fusion protein can be employed in various assay formats according to the present invention.

*Binding Assays*

The immunosuppressant assay reagent of the present invention can be employed in various assay formats for determining the presence or amount of immunophilin ligands or immunophilins in a test sample. In addition, where the immunophilin ligand is an immunosuppressive agent, the immunosuppressive assay reagent can be employed in an assay format for evaluating the immunosuppressive efficacy of such immunosuppressive agents in a test sample. The test sample can be any material containing an immunophilin ligand, and can be used directly as obtained from the source or following a pretreatment to modify the character of the sample. The test sample can be derived from any chemical or biological source, such as a physiological fluid, including but not limited to, whole blood, serum, plasma, saliva, ocular lens fluid, cerebral spinal fluid, sweat, urine, milk, ascites fluid, mucous, synovial fluid, peritoneal fluid, amniotic fluid, and the like, and fermentation broths, cell cultures, and chemical reaction mixtures. The test sample can be pretreated prior to use, such as preparing plasma from blood, diluting viscous fluids, and the like, to separate other components from the test

sample which may cause interference in the binding assay. For example, methods of pretreatment can involve filtration, distillation, centrifugation, concentration, inactivation of interfering components, and addition of reagents such as detergents for cell lysis and solubilization of an immunosuppressant binding protein, extraction with organic solvents, and the like. In some instances, it may be necessary to modify a solid test material to form a liquid medium or to release the immunophilin ligand.

In particular, the determination of immunophilin ligands or immunophilins and evaluation of immunosuppressive agents employing the immunosuppressant assay reagent of the present invention can be performed according to various specific binding assay formats known in the art including, but not intended to be limited to, homogeneous specific binding assay formats, heterogeneous assay formats, inhibition assay formats, heterogeneous immunoassay formats, homogeneous immunoassay formats, and the like, where the amount of a detectable reagent employed therein can be measured and correlated to the amount of an immunophilin ligand or immunophilin, or immunosuppressive activity of an immunosuppressive agent, in a test sample. According to such assay formats, the various reagent addition steps can be performed simultaneously or sequentially.

When performing such assay formats, the detectable reagent can be either the immunophilin ligand or analog thereof labeled with a detectable moiety, or an immunophilin or immunoreactant labeled with a detectable moiety. The detectable moiety can be any compound or conventional detectable chemical group having a detectable physical or chemical property which can be used to label an immunophilin ligand, an immunophilin, or an immunoreactant. Where an immunoreactant is employed, such as in a homogeneous or heterogeneous assay format, such immunoreactant can be a polyclonal or monoclonal antibody, a recombinant protein or recombinant antibody, a chimeric antibody, mixtures or fragments thereof, as well as a preparation of such antibodies, peptides and nucleotides for which suitability for use as specific binding members is well known to those skilled in the art.

Such detectable chemical groups include, but are not intended to be limited to, enzymatically active groups such as enzymes, enzyme substrates, prosthetic groups or coenzymes; spin labels; fluorescent molecules such as fluorescers and fluorogens; chromophores and chromogens; luminescent molecules such as chemiluminescers and bioluminescers; phosphorescent molecules; specifically bindable ligands such as biotin and avidin; electroactive species; radioisotopes;

toxins; drugs; haptens; DNA; RNA; polysaccharides; polypeptides; liposomes; colored particles and colored microparticles; and the like.

According to the present invention, homogeneous and heterogeneous assay formats depend upon the ability of (i) an immunophilin ligand or immunophilin in the presence of a detectable reagent comprising the immunophilin ligand or analog thereof, or an immunophilin, or an immunoreactant, labeled with a detectable chemical group, or (ii) in the case of an immunophilin, the ability of a detectable reagent comprising the immunophilin ligand or analog thereof labeled with a detectable chemical group, to specifically bind to the immunosuppressant binding protein component of the immunosuppressant assay reagent. For example, the extent of such binding is determined by the amount of the detectable chemical group present in the detectable reagent which either has or has not participated in a binding reaction with the immunophilin ligand, wherein the amount of the detectable reagent detected and measured can be correlated to the amount of the immunophilin ligand or immunophilin present in the test sample.

Homogeneous assays according to the present invention can be performed involving a competition between an immunophilin ligand from a test sample and a detectable reagent, comprising the immunophilin ligand or analog thereof labeled with a detectable chemical group, for a limited number of immunosuppressant binding protein sites of the immunosuppressant assay reagent. For example, immunophilin ligands can be determined according to the present invention in a fluorescent polarization assay employing a fluorescent detectable reagent, comprising the immunophilin ligand or analog thereof labeled with a fluorescent molecule, wherein the fluorescent detectable reagent, when excited by linearly polarized light, will emit fluorescence having a degree of polarization inversely related to its rate of rotation. When the fluorescent detectable reagent is bound to the immunosuppressant assay reagent and excited by linearly polarized polarized light, it is constrained from rotating between the time light is absorbed and emitted whereas, when the unbound fluorescent detectable reagent is excited by linearly polarized light, its rotation is much faster than the corresponding bound fluorescent detectable reagent and the molecules are more randomly orientated so that the emitted light is polarized. Accordingly, when plane polarized light is passed through a solution containing the aforementioned reagents, a fluorescent polarization response is detected and correlated to the amount of the immunophilin ligand present in the test sample. Various fluorescent compounds which can be employed for performing fluorescent polarization assays according to the present invention include, but are not intended to be limited to, aminofluoresceins, such as

described in U.S. Patent No. 4,510,251 and U.S. Patent No. 4,614,823, incorporated herein by reference; triazinylaminofluoresceins, such as described in U.S. Patent No. 4,420,568 and U.S. Patent No. 4,593,089, incorporated herein by reference; carboxyfluoresceins, such as described in U.S. Patent No. 4,668,640, incorporated herein by reference; and the like.

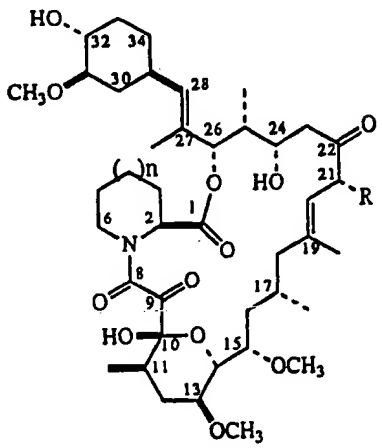
Similarly, heterogeneous immunoassays according to the present invention can be performed involving binding reactions among an immunophilin ligand from a test sample, the immunosuppressant assay reagent of the present invention, and a detectable reagent comprising an immunophilin or immunoreactant for the immunophilin ligand labeled with a detectable chemical group, wherein the extent of binding of the detectable reagent to the immunophilin ligand is a function of the amount of the immunophilin ligand present in the test sample. In order to correlate the amount of detectable reagent bound to the immunophilin ligand to the amount of the immunophilin ligand present in the test sample, any of the detectable reagent not bound to the immunophilin ligand must be separated therefrom. Such separation can be accomplished employing solid phase materials for the direct immobilization of the immunosuppressant assay reagent thereto according to methods known in the art such as adsorption techniques, covalent binding techniques, and the like. The solid phase materials can be any solid material to which the immunosuppressant assay reagent can be immobilized and include, but are not intended to be limited to, beads, magnetic particles, micro or macro particles, test tubes, and microtiter plates. Such solid phase materials can be made from synthetic materials, naturally occurring materials, or naturally occurring materials which have been synthetically modified, and include, but are not intended to be limited to, cellulose materials, such as paper, cellulose and cellulose derivatives such as cellulose acetate and nitrocellulose; fiberglass; naturally occurring cloth such as cotton; synthetic cloth such as nylon; porous gels, such as silica, agarose, dextran, and gelatin; porous fibrous matrixes; starch based materials, such as cross-linked dextran chains; ceramic materials; olefin or thermoplastic materials including polyvinyl chloride, polyethylene, polyvinyl acetate, polyamide, polycarbonate, polystyrene, copolymers of vinyl acetate and vinyl chloride, combinations of polyvinyl chloride-silica; and the like.

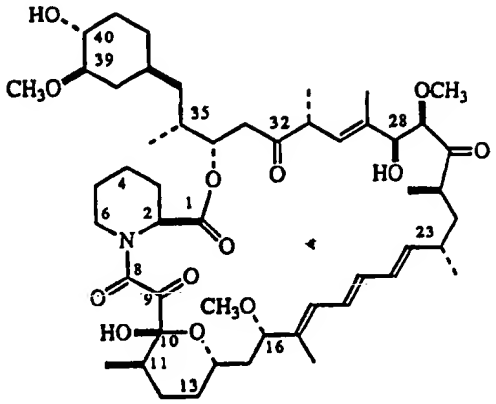
For example, a heterogeneous assay format which can be performed according to the present invention is a competitive format wherein the immunosuppressant assay reagent is immobilized to a solid phase material whereby upon separation, the amount of detectable reagent, comprising the immunophilin ligand or analog thereof labeled with a detectable chemical group, which is bound

to such solid phase material can be detected and correlated to the amount of the immunophilin ligand present in the test sample. Another form of a heterogeneous assay format which can be performed according to the present invention is a sandwich assay format which involves contacting a test sample containing an immunophilin ligand with an immobilized form of the immunosuppressant assay reagent as described above, wherein the immunophilin ligand binds to the immobilized immunosuppressant assay reagent to form a complex therewith. Such complex is then contacted with a detectable reagent comprising an immunophilin or immunoreactant for the immunophilin ligand, labeled with a detectable chemical group, and, following, for example, one or more washing steps to remove any unbound material, the detectable reagent bound to the complex is measured and correlated to the amount of the immunophilin ligand present in the test sample.

Various detectable reagents of the present invention can be prepared employing FK-506 and analogs thereof, such as FK-523 and FK-525. FK-506 and analogs thereof can be isolated, for example, from culture media obtained according to methods known in the art by fermentation of microorganisms of the genus *Streptomyces*, such as described in European Patent Application No. 0184162, available from the Fermentation Research Institute, Tsukuba, Ibaraki 305, Japan under the provisions of the Budapest Treaty, under deposit No. FERM BP-927 (redeposited on April 27, 1989 with the Agricultural Research Culture Collection International Depository, Peoria, Illinois 61604, USA under the provisions of the Budapest Treaty, under deposit No. NRRL 18488). In addition, the macrolide FR-900520 (European Patent Application 0184162), also known as ascomycin, may be prepared according to methods known in the art [H. Hatanaka, M. Iwami, T. Kino, T. Goto and M. Okuhara, FR-900520 and FR-900523, *Novel immunosuppressants isolated from A streptomyces. I. Taxonomy of the producing strain* , J. Antibiot., 1988, XLI(11), 1586-1591; H. Hatanaka, T. Kino, S. Miyata, N. Inamura, A. Kuroda, T. Goto, H. Tanaka and M. Okuhara, FR-900520 and FR-900523, *Novel immunosuppressants isolated from A streptomyces. II. Fermentation, isolation and physico-chemical and biological characteristics*, J. Antibiot., 1988. XLI(11), 1592-1601; T. Arai, Y. Koyama, T. Suenaga and H. Honda, *Ascomycin, An Antifungal Antibiotic*, J. Antibiot., 1962, 15(231-2); and U.S. Patent No. 3,244,592]. Furthermore, rapamycin can be produced by *Streptomyces hygrosopicus*, using conditions adapted from the literature [C. Vezina, A. Kudelski, S. N. Sehgal, J. Antibiot., 28, 721-6 (1975); S. N. Sehgal, H. Baker, C. Vezina, Journal of Antibiotics, 28, 727-32 (1975)].

The chemical structures of FK-506, analogs of FK-506 and rapamycin are represented by the following general formulae:





FK-506: n=1, R= allyl
Ascomycin (FR-900520): n=1, R= ethyl
FK-523: n=1, R= methyl
FK-525: n=0, R= allyl
Dihydro-FK-506: n=1, R= propyl

Rapamycin

When performing a specific binding assay for an immunophilin ligand according to the present invention, a preferred detectable reagent comprises an immunosuppressive agent labeled with a hydrolytic enzyme such as alkaline phosphatase or horseradish peroxidase. For example, ascomycin, an analog of FK-506, and which can be used in a specific binding assay for FK-506, can be converted to its C22 O-carboxymethyloxime using O-carboxymethyl hydroxylamine (Figure 13). After conversion to the oxime, an activated ester can be prepared by reaction with N-hydroxysuccinimide and dicyclohexyl carbodiimide. Reaction of the activated ester with alkaline phosphatase yields an ascomycin-alkaline phosphatase detectable reagent. The ascomycin-alkaline phosphatase detectable reagent can catalyze the hydrolysis of various substrates to produce detectable signals, such as catalysis of p-nitrophenyl phosphate to produce a colorimetric signal; catalysis of 4-methyl umbelliferyl phosphate to produce a fluorimetric signal; catalysis of water-soluble 1,2-dioxetane derivatives (Tropix, Inc., Bedford, MA) to produce a chemiluminescent signal, and the like. A radioactive detectable reagent, [$^3$H]-dihydro-FK-506, can be prepared by catalytic reduction of FK-506 using palladium on carbon and exposure to tritium gas, and subsequent purification of the radiolabelled compound can be achieved by thin layer chromatography or HPLC.

As shown in Figure 3, when performing a heterogeneous assay employing a solid phase material as described above, the present inventors have surprisingly and unexpectedly found that the fusion of the heterologous protein to the immunosuppressant binding protein results in the retention of superior binding activity of the immunosuppressant binding protein when immobilized to a solid phase material. On the other hand, the native form of the immunosuppressant binding protein lacks such superior binding activity when immobilized to a solid phase material. Another method by which an immunosuppressant assay reagent can be bound to a solid phase phase material is through the use of an immunoreactant as described above, such as an antibody (Figure 11) which is immobilized to the solid phase material according to methods known in the art and which binds to a region of the heterologous protein distant from the active site or desired antigenic site of the immunosuppressant binding protein. Such "passive antibody" coupling technique also serves to conserve reagents. It is to be understood that the immunosuppressant assay reagent can be immobilized to the solid phase material prior to performance of the binding assay or formed *in situ* during the course of performing the binding assay. In addition, the immunosuppressant assay reagent can be immobilized to a solid phase material by derivatizing the immunosuppressant assay reagent with a first binding member of a specific binding member pair, and coupling or linking a second binding member of the specific binding member pair to the solid phase material, wherein immobilization of the immunosuppressant assay reagent is achieved by binding of the first and second binding members. Such binding pairs as contemplated herein are two different molecules wherein one of the molecules specifically binds to the second molecule through chemical or physical means. Such binding pairs include, but are not intended to be limited to, analytes and antibodies, biotin and avidin, carbohydrates and lectins, complementary nucleotide sequences, complementary peptide sequences, effector and receptor molecules, enzyme cofactors and enzymes, enzyme inhibitors and enzymes, a peptide sequence and an antibody specific for the sequence or the entire protein, polymeric acids and bases, dyes and protein binders, peptides and specific protein binders (e.g., ribonuclease, S-peptide and ribonuclease S-protein), and the like. Furthermore, binding pairs can include members that are analogs of the original binding member, for example, an analyte-analog or a binding member made by recombinant techniques or molecular engineering, or an immunoreactant as defined above.

Another method by which the immunosuppressant assay reagent can be immobilized to a solid phase material is through chemical activation of the solid

phase material in order to couple the immunosuppressant assay reagent thereto. For example, Sepharose™ resin particles (Pharmacia) can be activated with cyanogen bromide or diols by sodium periodate, or with free amines by formaldehyde, glutaraldehyde, and the like, wherein the immunosuppressant assay reagent irreversibly binds to such reactive groups.

It is to be understood that according to the present invention, the detectable reagent can be one which is capable of binding to the immunosuppressant assay reagent-immunophilin ligand complex while, at the same time, is capable of providing a detectable signal. In particular, according to a preferred embodiment, the detectable reagent is a calcium/calmodulin activated phosphatase, such as calcineurin, which is regulated by the calcium ion and calmodulin. According to this embodiment, immunosuppressive agents such as FK-506 and analogs thereof are detected by their ability to enhance the binding of calcineurin to the immunosuppressant binding protein of the immunosuppressant assay reagent. For example, a binding assay for FK-506 and analogs thereof can be performed employing calcineurin and the immunosuppressant assay reagent of the present invention as described in Example 13. According to such assay format the immunosuppressant assay reagent is immobilized to a solid phase material, such as the well walls of a microtiter plate, and a test sample containing an immunophilin ligand, particularly an immunosuppressive agent such as FK-506, and calcineurin are added thereto. A substrate for calcineurin, such as para-nitrophenyl phosphate, is added wherein upon reacting with calcineurin, produces para-nitrophenol (a yellow product), and the temporal change in 405 nm absorbance is measured and correlated to the amount of the immunosuppressive agent in the test sample. As shown in Figure 8, some macrolide immunosuppressants such as FK-506 enhance the binding of calcineurin to the FKBP-CKS immunosuppressant assay agent and; therefore, increase the amount of para-nitrophenol product produced therefrom. However, other macrolide immunosuppressants, like rapamycin, are unable to enhance calcineurin binding to the FKBP-CKS and can not be directly measured with this method.

Figure 9 illustrates the ability of FK-506 and ascomycin to increase, in a concentration dependent manner, the amount of signal produced when such macrolide immunosuppressive agents are included in the assay format of Example 13. FK-506 and ascomycin are detected when they are present in the assay at concentrations greater than 1 nM. Since the level of signal is related to the concentration of the macrolide immunosuppressive agent, standard curves can be created to relate the known concentration of a purified FK-506 to the amount of

signal produced and used to estimate the unknown amount of the FK-506 in a sample.

An assay for rapamycin and structurally similar molecules can also be performed based upon their ability to compete for FK-506 binding to the FKBP-CKS immunosuppressant assay reagent. In this configuration, the assay is performed as described in Example 13 with the inclusion of a standard concentration of FK-506, preferably 10 nM, in the macrolide solution that contains rapamycin or a rapamycin-like molecule. Due to rapamycin's ability to compete for the binding of FK-506 to the FKBP-CKS immunosuppressant assay reagent without binding calcineurin, the amount of calcineurin bound to the immobilized FKBP-CKS immunosuppressant assay reagent in the wells is reduced in relation to the concentration of rapamycin. A standard curve can be created to relate the known concentration of a purified rapamycin-like competitor to the amount of signal produced by the detectable reagent and used to estimate the unknown amount of the rapamycin-like competitor in a sample.

Other molecules, known as competitors, can also bind to the immunosuppressant assay reagent wherein when binding thereto, they can prevent binding of the immunophilin ligand of interest and thereby reduce the amount of signal produced by the detectable reagent when standard amounts of the immunophilin ligand and the immunosuppressant assay reagent are coincubated with a test sample containing the competitor molecule. Quantitation of an unknown amount of competitor molecule in a test sample can be achieved by comparing the effect of the test sample and the effect of known amounts of the competitor molecule on the level of signal produced by the detectable reagent when they are coincubated with standard amounts of the immunosuppressant binding reagent and the immunophilin ligand. For example, immunosuppressive agents such as rapamycin and analogs thereof can be detected by their ability to inhibit the binding of calcineurin to the immunosuppressant binding protein of the immunosuppressant assay reagent when FK-506 and analogs thereof are also present. Quantitation of the amount of immunosuppressive agent in the test sample can be accomplished by comparison to a standard curve that relates the amount of calcineurin bound when known amounts of the immunosuppressive agent of interest is added to the assay format.

The immunosuppressant assay reagent of the present invention can also be used to determine the presence or amount of a immunosuppressant binding protein in order to aid in the prediction of efficacious therapeutic doses of an immunosuppressive agent and individual related toxicity. Such determination can

be made in an inhibition assay format comprising a test sample containing the immunosuppressant binding protein of interest, an immobilized form of the immunosuppressant assay reagent of the present invention, and a detectable reagent comprising the immunophilin ligand or analog thereof labeled with a detectable moiety, wherein the immunophilin ligand is capable of binding to the immunosuppressant binding protein from the test sample. According to such assay format, binding of the immunosuppressant binding protein from the test sample to the detectable reagent will inhibit binding of the detectable reagent to the immobilized immunosuppressant assay reagent, wherein the amount of labeled reagent bound to the immobilized immunosuppressant assay reagent can be measured and correlated to the amount of the immunosuppressant binding protein present in the test sample. For example, Figure 10 illustrates the results of such inhibition assay format for the determination of FKBP wherein a FKBP-CKS immunosuppressant assay reagent immobilized to microtiter plate well walls was reacted with a detectable reagent comprising ascomycin labeled with alkaline phosphatase in the presence of increasing amounts of FKBP in the microtiter wells. Any of the detectable reagent which was not bound to the immobilized immunosuppressant assay reagent was washed from the wells, para-nitrophenyl phosphate was added to the wells, and the absorbance of the reaction between the substrate and the alkaline phosphatase was measured at 405 nm. It is to be understood that when making such determination, it may be necessary to treat the test sample, particularly where the test sample is, for example, whole blood, with a detergent in order to solubilize the immunosuppressant binding protein contained therein.

*Purification Methods*

The immunosuppressant assay reagent can be used for isolation and purification of immunophilin ligands by immobilizing the immunosuppressant assay reagent of the present invention to a solid support material, such as in a column format, and contacting the immunosuppressant assay reagent with a test sample. The immunophilin ligand can be recovered from the immunobilized immunosuppressant assay reagent by conditions which disrupt the binding interaction between the immunosuppressant assay reagent and the immunophilin ligand. For example, such binding interaction can be disrupted by raising or lowering the pH or by the use of protein denaturants such as urea, guanidinium, hydrochloric acid, sodium dodecyl sulfate, and the like.

*Test Kit*

A test kit according to the present invention comprises all of the essential reagents required to perform a desired specific binding assay for an immunophilin ligand or immunophilin, or for the evaluation of the immunosuppressive activity of an immunosuppressive agent, as described herein. The test kit is presented in a commercially packaged form as a combination of one or more containers holding the necessary reagents, as a composition or admixture where the compatibility of the reagents will allow. Particularly preferred is a test kit for the determination of macrolide immunosuppressive agents in a heterogeneous binding assay format and wherein the immunosuppressant assay reagent comprises an immunosuppressant binding protein and CKS immobilized to a solid support material as described herein. It is to be understood that the test kit can, of course, include other materials as are known in the art and which may be desirable from a commercial user standpoint, such as buffers, diluents, pretreatment reagents, and the like.

The present invention will now be illustrated, but is not intended to be limited, by the following examples.

Example 1

Preparation of Native FKBP And FKBP-CKS Immunosuppressant Assay Reagent

(a) Native (non-fusion) FKBP was produced by inoculating a single colony of *E. coli* in 35 mL of LB broth containing 50 ug/ml ampicillin and 12.5 ug/ml tetracycline in a 250 mL flask. The flask was incubated at 37°C while centrifuging at 225 rpm until early stationary phase, and then inoculated into 10 liters of M9 media (0.2% glucose, 2% Difco casamino acids, 0.1 % polyproplyene glycol 2000 [anti-foam; Aldrich Chemical Co., Milwaukee, Wisconsin], trace metals [Obukowicz, et al., Biochem., Volume 29, pages 9737-97-45 (1990)], 50 ug/ml ampicillin and 12.5 ug/ml tetracycline in a BioLafette BL15.2 15 liter fermenter. The cells were grown at 37°C while centrifuging at 300 rpm with 10 liters/minute air. The pH was maintained at 7.0 by automatic addition of 4N KOH or 4N acetic acid. Glucose was maintained at approximately 0.1-0.2% by manual addition of 60% glucose. The $pO_2$ was maintained between 10-50% by manually increasing the agitation rate. The culture was induced at an $A_{600}$ of 10 by the addition of 0.5 mM IPTG. After an addition 10 hours of growth, the culture was harvested (the final $A_{600}$ was 15). The cells were lysed with 3-4 ml/gram of cells

with a lysis buffer consisting of 50 mM phosphate, 10 mM EDTA, 10 mM magnesium chloride, pH 7.4, containing freshly added lysozyme (Sigma Chemical Co., St. Louis, MO) at 0.5 mg/ml with 5 mM DTT (U.S. Biochemicals, Cleveland, OH), 1 ug/ml of DNAase (Boehringer Mannheim, Indianapolis, IN), and 1 mM PMSF (Sigma Chemical Co.). The cells were stirred at room temperature for 40-60 minutes followed by sonication on an ice bath with 1 minute pulses for 5-10 minutes, and the cells were checked for lysis on a microscope and centrifuged for 40 minutes at 9000 rpm in a GS3 Sorvall rotor. The resultant lysant was subjected to 40-60% fractionation at 4°C and the precipitate was pelleted by centrifugation. The pellet was placed in dialysis tubing (Spectra/pore™ 6000-8000 MWCO, Spectrum Medical Ind., Los Angeles, CA) and dialyzed against three 2 liter changes of 10 mM Hepes, 0.5 mM DTT, pH 8.0, at 4°C. The dialyzed protein was applied to fast flow Q-Sepharose™ column,(4.5 x 41 cm) pre-equilibrated in dialysis buffer. The column was developed isocratically at 1 ml/minute and the native FKBP was eluted as the first peak, and the purified protein was pooled and precipitated by addition of ammonium sulfate to 80% saturation. The resulting precipitate was pelleted by centrifugation and dialyzed as described above, and stored in the presence of 0.004% sodium nitrate. The purified protein was expressed as a non-fusion construct estimated at 5-10 mg per liter of harvested culture media.

(b) An immunusuppressant assay reagent of the present invention comprising FKBP and CKS was prepared by isolating human FKBP from a Jurkat T cell cDNA library and incorporating it into an *E. coli* expression vector containing the CKS gene under the control of a *lac* promoter. The DNA for FKBP-CKS fusion protein was prepared from the FKBP gene by cloning into an expression vector containing the CKS gene under the control of a modified *lac* promoter as described by copending U.S. Patent Application Serial No. 276,263, filed November 23, entitled "CKS Method Of Protein Synthesis and by T. Bolling and W. Mandecki, Biotechniques, Volume 8, pages 488-490 (1990). The fusion protein preparation was obtained from lysed *E. coli* by 25 to 35% ammonium sulfate fractionation.

## Example 2

### Ascomycin-Protein Conjugates (position 22)

(a) Ascomycin-C22-carboxymethyloxime (Figure 13, Compound II, R = ethyl, n = 1).

A stirred solution of ascomycin (1.26 mmol, 1.0 g), carboxymethoxylamine hemihydrochloride (1.52 mmol, 0.33 g) and N-methylmorpholine (2.78 mmol, 0.31 mL) in ethanol (5 mL) was heated at 80° C for one hour, cooled to room temperature, and evaporated. The reaction mixture was partitioned between ethyl acetate and 0.1 M H3PO4, and the ethyl acetate layer was washed with brine, dried over Na2SO4, filtered, and concentrated to give 1.5 g of crude product. The crude product was redissolved in dichloromethane (20 mL), filtered through a silica gel plug (20 mL), and eluted with 30% acetone/hexane (2 x 50 mL) to remove unreacted starting material, then 50% acetone/hexane (4 x 50 mL) to give 650 mg of the desired product in 60% yield.

(b) Ascomycin-C22-Carboxymethyloxime-BSA Conjugate (Figure 13, Compound III, R = ethyl, n = 1, NH-X = BSA).

To a stirred solution of ascomycin-C22-carboxymethyloxime (100 mg, 0.116 mmol) in dry dimethylformamide (2.3 mL) were added hydroxysuccinimide (13.3 mg) and dicyclohexylcarbodiimide (23.8 mg) sequentially. After 15.5 h the solids were removed, and the supernatant containing the resulting crude active ester was used directly in the coupling procedure described below. BSA (25 mg, Sigma Chemical Co.) was dissolved in 2 mL of 50 mM sodium phosphate buffer, pH 6, and mixed with the ascomycin active ester prepared above (10 umol, 0.20 mL of above dimethylformamide solution). After mixing for 4 days at 4°C, the mixture was dialyzed against 50 mM sodium phosphate buffer, pH 7, to give the desired ligand/protein conjugate.

(c) Ascomycin-C22-carboxymethyloxime-KLH conjugate (Figure 13, Compound III, R = ethyl, n = 1, NH-X = KLH) was prepared according to the procedure of this Example 2b by replacing BSA with KLH (Sigma Chemical Co.).

(d) Ascomycin-C22-carboxymethyloxime-alkaline phosphatase conjugate (Figure 13, Compound III, R = ethyl, n = 1, NH-X = alkaline phosphatase) was prepared according to the procedure of this Example 2b by replacing BSA with 22 mg of alkaline phosphatase (Abbott Laboratories, Abbott Park, Illinois) and using 2 umol of the ascomycin active ester in 0.2 ml of dimethylformamide.

Example 3

Synthesis Of FK-506-Protein Conjugates (position 22)

(a) FK-506-C22-carboxymethyloxime (Figure 13, Compound II, R = allyl, n = 1) is prepared according to the procedure of Example 2a by replacing ascomycin with FK-506.

(b) FK-506-C22-carboxymethyloxime-BSA conjugate (Figure 13, Compound III, R = allyl, n = 1, NHX = BSA) is prepared according to the procedure of Example 2b by replacing ascomycin-C22-carboxymethyloxime with the resultant product of this Example 3a.

(c) FK-506-C22-carboxymethyloxime-KLH conjugate (Figure 13, Compound III, R = allyl, n = 1, NHX = KLH) is prepared according to the procedure of this Example 3b by replacing BSA with KLH (Sigma Chemical Co.).

(d) FK-506-C22-carboxymethyloxime-alkaline phosphatase Conjugate (Figure 13, Compound III, R = allyl, n = 1, NHX = alkaline phosphatase) is prepared according to the procedure of this Example 3b by replacing BSA with alkaline phosphatase.

Example 4

Synthesis Of FK-523-Protein Conjugates (position 22)

(a) FK-523-C22-carboxymethyloxime (Figure 13, Compound II, R = methyl, n = 1) is prepared according to the procedure of Example 2a by replacing ascomycin with FK-523.

(b) FK-523-C22-carboxymethyloxime-BSA conjugate (Figure 13, Compound III, R = methyl, n = 1, NHX = BSA) is prepared according to the procedure of Example 2b by replacing ascomycin-C22-carboxymethyloxime with the resultant product of Example 4a.

(c) FK-523-C22-carboxymethyloxime-KLH Conjugate (Figure 13, Compound III, R = methyl, n = 1, NHX = KLH) is prepared according to the procedure of this Example 4b by replacing BSA with KLH (Sigma Chemical Co.).

(d) FK-523-C22-carboxymethyloxime-alkaline phosphatase Conjugate (Figure 13, Compound III, R = methyl, n = 1, NHX = alkaline phosphatase) is prepared according to the procedure of this Example 4b by replacing BSA with alkaline phosphatase.

Example 5

Synthesis of FK-525-Protein Conjugates (position 22)

(a) FK-525-C22-carboxymethyloxime (Figure 13, Compound II, R = allyl, n = 0) is prepared according to the procedure of Example 2a by replacing ascomycin with FK-525.

(b) FK-525-C22-carboxymethyloxime-BSA conjugate (Figure 13, Compound III, R = allyl, n = 0, NHX = BSA) is prepared according to the procedure of Example 2b by replacing ascomycin-C22-carboxymethyloxime with the resultant product of Example 3a.

(c) FK-525-C22-carboxymethyloxime-KLH conjugate (Figure 13, Compound III, R = allyl, n = 0, NHX = KLH) is prepared according to the procedure of this Example 5b by replacing BSA with KLH (Sigma Chemical Co.).

(d) FK-525-C22-carboxymethyloxime-alkaline phosphatase Conjugate (Figure 13, Compound III, R = allyl, n = 0, NHX = Alkaline Phosphatase) is prepared according to the procedure of this Example 5b by replacing BSA with alkaline phosphatase.

Example 6

Synthesis Of Dihydro-FK506 -Protein Conjugates (position 22)

(a) Dihydro-FK506 (Figure 13, Compound I, R = propyl, n = 1) was prepared by hydrogenating FK-506 (150 mg) and 10% Pd/C (30 mg) in ethylacetate (6 ml) at room temperature for 20 min at 1 atm. The catalyst was filtered, the solvent was concentrated under vacuum, and the resulting crude material was purified by silica gel column chromatography (eluting solvent, chloroform/acetone 5:1) to give the title compound.

(b) Dihydro-FK506-C22-carboxymethyloxime (Figure 13, Compound II, R = propyl, n = 1) is prepared according to the procedure of Example 2a by replacing ascomycin with the dihydro-FK506.

(c) Dihydro-FK506-C22-carboxymethyloxime-BSA conjugate (Figure 13, Compound III, R = propyl, n = 1, NHX = BSA) is prepared according to the procedure of Example 2b by replacing ascomycin-C22-carboxymethyloxime with the resultant product of this Example 6b.

(d) Dihydro-FK506-C22-carboxymethyloxime-KLH conjugate (Figure 13, Compound III, R = propyl, n= 1, NHX = KLH) is prepared according

to the procedure of this Example 6c by replacing BSA with KLH (Sigma Chemical Co.).

(e) Dihydro-FK506-C22-carboxymethyloxime-alkaline phosphatase conjugate (Figure 13, Compound III, R = propyl, n = 1, NHX = Alkaline Phosphatase) is prepared according to the procedure of this Example 6c by replacing BSA with alkaline phosphatase.

Example 7

Synthesis Of Carboxymethyloxime Rapamycin-Protein Conjugates (position 32)

(a) Rapamycin-C32-Carboxymethyloxime (position 32, Figure 14, Compound V) is prepared by stirring a solution of rapamycin (1.26 mmol, 1.15 g), carboxymethoxylamine hemihydrochloride (1.52 mmol, 0.33 g) and N-methylmorpholine (2.78 mmol, 0.31 mL) in ethanol (5 mL), and allowed to react until the reaction was judged complete by TLC. The reaction mixture was evaporated, partitioned between ethyl acetate and 0.1 M H3PO4, and the ethyl acetate layer was washed with brine, dried over Na2SO4, filtered, and concentrated to give crude product which was purified by recrystallization.

(b) Rapamycin-C32-carboxymethyloxime-BSA conjugate (position 32, Figure 14, Compound VI, NH-Y = BSA) is prepared according to the procedure of Example 2b, by replacing ascomycin-C22-carboxymethyloxime with the resultant product of this Example 7a.

(c) Rapamycin-C32-carboxymethyloxime-KLH conjugate (position 32, Figure 14, Compound VI, NH-Y = KLH) is prepared according to the procedure of this Example 7b by replacing BSA with KLH (Sigma Chemical Co.).

(d) Rapamycin-C32-carboxymethyloxime-alkaline phosphatase conjugate (position 32, Figure 13, Compound VI, NH-Y = alkaline phosphatase) is prepared according to the procedure of this Example 7b by replacing BSA with alkaline phosphatase.

Example 8

Synthesis Of [$^3$H]-Dihydro-FK506

(Figure 13, Compound I, R= $CH_2C^3HH_2C^3HH_2$, n = 1)

[$^3$H]-Dihydro-FK506 was prepared according to the procedure of Example 6a by replacing hydrogen gas with tritium gas.

Example 9

Comparison of FKBP-CKS Immunoassay Reagent to FKBP for Binding of Ascomycin-Alkaline Phosphatase Conjugate

A binding assay for FK-506 was performed employing an immobilized form of the FKBP-CKS immunosuppressant assay reagent as described in Example 1 and an ascomycin-alkaline phosphatase conjugate as described in Example 2d.. A solution of the FKBP-CKS immunosuppressant assay reagent as described in Example 1 was prepared at a concentration of 10-35 µg/ml in 20 mM sodium phosphate buffer, pH 7.4, and 100 µl was incubated in the wells of a Immuno Plate MaxiSorp™ plate (Nunc, Naperville, IL) at ambient temperature for 2 hours in order to adsorb the immunosuppressant assay reagent to the walls of the wells. A solution of phosphate buffered saline (PBS), pH 7.4, containing 2% bovine serum albumin (BSA) and 0.2 % Tween 20 was then added to the wells and incubated for 30 minutes at ambient temperature.

The wells were then emptied and rinsed with 0.2 % Tween 20 in PBS. Fifty micoliters of a solution containing FK-506, or other inhibitors, in the PBS/BSA/Tween 20 buffer, as well as buffer alone, was added to the wells. An equal volume of solution containing the ascomycin-alkaline phosphatase conjugate at a concentration of 0.3-5 µg/ml in PBS/BSA/Tween 20 waaso added to the wells and incubated for 2 hours at ambient temperature. The wells were then emptied and rinsed with 0.2 % Tween 20 in PBS, and a solution of para-nitrophenyl phosphate at a concentration of 1 mg per ml in 0.1 M aminomethyl propanol was added to the wells and the temporal change in 405 nm absorbance was measured.

The temporal change in 405 nm absorbance was related to the amount of conjugate bound to the immobilized immunosuppressant assay reagent in the wells wherein a yellow product, para-nitrophenol, was produced as a result of the interaction of the alkaline phosphatase and para-nitrophenyl phosphate. The FK-506 blocked the binding of conjugate to the FKBP-CKS immunosuppressant assay reagent to thereby reduce the amount of para-nitrophenol product. Total binding of the conjugate was determined from the amount of signal produced when no FK-506 was present in the solution. Nonspecific binding of the conjugate was determined from the amount of signal produced when 1-10 µM of FK-506, rapamycin, or ascomycin are present in the sample.

Shown in Figure 2 is a comparison of the amount of para-nitrophenol product formed, change in 405 nm absorbance, when 3.3 µg of the FKBP/CKS immunoassay reagent or FKBP was used to coat the wells of the plate in this Example 9. The Total represents the results when 5 µg/ml of ascomycin-alkaline

phosphatase was incubated in the wells without an inhibitor present. To determine the Nonspecific binding of the conjugate, 10 μM ascomycin was added to the wells with the ascomycin-alkaline phosphatase conjugate. The use of FKBP/CKS resulted in a higher signal-to-noise, greater difference between the Total and Nonspecific binding, as compared to when FKBP is used.

As shown in Figure 3, FK-506 and rapamycin reduced, in a concentration dependent manner, the amount of signal produced when added to the assay of Example 9 when the wells were coated with 3 μg of FKBP-CKS immunoassay reagent and 0.5 μg/ml of ascomycin-alkaline phosphatase conjugate was used. Wherein FK-506 and rapamycin concentration as low as 0.2 nM can be detected in such assay. Accordingly, standard curves, relating the known concentration of a purified immunosuppressive agent to the amount of signal produced, can be created and used to estimate the amount of the immunosuppressive agent in a sample.

## Example 10

### Binding Of [$^3$H]-Dihydro-FK-506 To FKBP-CKS Immunosuppressant Assay Reagent

A binding assay for [$^3$H]-dihydro-FK-506 was performed employing an immobilized form of the FKBP-CKS immunosuppressant assay reagent of the present invention. A solution of the FKBP-CKS immunosuppressant assay reagent as described in Example 1 was prepared at a concentration of 10-35 μg/ml in 20 mM sodium phosphate buffer, pH 7.4, and incubated in the wells of a Immuno Plate MaxiSorp™ plate (Nunc, Naperville, IL) at ambient temperature for 2 hours in order to adsorb the immunosuppressant assay reagent to the walls of the wells. A solution of phosphate buffered saline (PBS), pH 7.4, containing 2 % bovine serum albumin (BSA) and 0.2 % Tween 20 was then added to the wells and incubated for 30 minutes at ambient temperature to reduce the nonspecific binding of [$^3$H]-dihydro-FK-506 to the well walls as described below. The wells were then emptied and rinsed with 0.2 % Tween 20 in PBS, and a solution containing FK-506 in the PBS/BSA/Tween 20 buffer, as well as buffer alone, were added to the wells. An equal volume of a solution containing [$^3$H]-dihydro-FK-506 in PBS/BSA/Tween 20, was added to the wells and incubated for 2 hours at ambient temperature, and the wells emptied and rinsed with 0.2 % Tween 20 in PBS. The radioactivity of the [$^3$H]-dihydro-FK-506 bound to the immobilized

FKBP-CKS immunosuppressant assay reagent in the wells was then determined by liquid scintillation spectrometry.

Total binding of the [$^3$H]-dihydro-FK-506 was determined from the amount of signal produced when no FK-506 was present in the test sample. Nonspecific binding of the [$^3$H]-dihydro-FK-506 was determined from the amount of signal produced when 1-10 μM of FK-506, rapamycin, or ascomycin were present in the sample. A 1.4 nM concentration of [$^3$H]-dihydro-FK-506 was incubated in the wells in the absence (Total) and presence (Nonspecific) of 4 μM ascomycin. The results of [$^3$H]-dihydro-FK-506 binding to the immobilized FKBP-CKS immunosuppressant assay reagent are shown in Figure 4. Standard curves as described in Example 9, relating the known concentration of a purified competitor to the amount of radioactive signal produced, can be created and used to determine the amount of the competitor in the test sample.

Example 11

Binding of A Multicomponent Ligand System Using Ascomycin-BSA Conjugate To the FKBP-CKS Immunosuppressant Assay Reagent

An assay format employing a multicomponent binding reagent system comprising (i) ascomycin conjugated to multiple sites on a macromolecule, such as bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), thyroglobulin, and the like (ii) an antiserum containing a first antibody which binds the ascomycin-macromolecule conjugate, and (iii) a second antibody which can bind to the first antibody to produce a detectable signal, was performed employing the following reagents:.

(a) Ascomycin was coupled to BSA and KLH by reaction as described in Example 2b and 2c, respectively..

(b) Rabbit antiserum containing antibodies to the ascomycin-KLH conjugate were prepared by immunization according to methods known in the art. Six mg of the ascomycin-KLH conjugate in 5 ml of phosphate-buffered physiological saline was emulsified in 5 ml of complete Freund's adjuvant. One ml of the emulsion was used to immunize each of three New Zealand white rabbits by injection intraperitoneally, intramuscularly, and intradermally. After 5 1/2 weeks, the rabbits were immunized by injection as before with an equal amount of the ascomycin-KLH conjugate emulsified in incomplete Freund's adjuvant. Two weeks after the booster injection, blood samples were collected from the ear vein.

Serum was obtained by centrifugation after allowing the blood to clot at 37°C for 60 minutes.

(c) Goat anti-rabbit IgG antibodies, conjugated with alkaline phosphatase (available from Sigma Chemical Co.) were employed as the second antibody in the multicomponent binding reagent system.

For performance of the assay, the FKBP-CKS immunosuppressant assay reagent as described in Example 1 was dissolved at a concentration of 10-20 µg/ml in 20 mM sodium phosphate buffer, pH 7.4, and added to the wells of a polystyrene microtiter plate and incubated at ambient temperature for 2 hours in order to immobilize the the immunosuppressant assay reagent to the well walls.. To reduce the nonspecific binding of other proteins, a solution of phosphate buffered saline (PBS), pH 7.4, containing 2 % bovine serum albumin (BSA) and 0.2 % Tween 20 was added to the wells and incubated for 30 minutes at ambient temperature. The wells were emptied and rinsed with 0.2 % Tween 20 in PBS, and 50 µl of a solution containing FK-506, or other inhibitor, in the PBS/BSA/Tween 20 buffer was then added to the wells. An equal volume of solution containing the ascomycin-BSA conjugate, diluted 1:150,000 in PBS/BSA/Tween 20, was also added to the wells and incubated for 90 minutes at 37°C. The wells were emptied and rinsed with PBS/Tween 20, and 100 µl of a solution containing the rabbit anti-ascomycin antiserum in PBS/BSA/Tween 20 was then added and incubated for 90 minutes at 37°C. The wells were emptied and rinsed with PBS/Tween 20, and 100 µl a solution containing the goat anti-rabbit IgG antiserum conjugated to alkaline phosphatase (diluted as recommended by the manufacturer) was then added and incubated for 90 minutes at 37°C. The wells were emptied and rinsed with PBS/Tween 20, and a solution of para-nitrophenyl phosphate (1 mg per ml in 0.1 M aminomethyl propanol) was then added to the wells and the temporal change in 405 nm absorbance was recorded.

As shown in Figures 5 and 6, the immobilized FKBP-CKS immunosuppressant assay reagent binds the multicomponent ligand system ("total" in Figure 5) in a manner which was inhibited by the presence of a sufficient concentration (1 µM) of FK-506 ("nonspecific" in Figure 5). Figure 6 illustrates the effect of adding increasing concentrations of FK-506, rapamycin, and ascomycin in an assay format as described in Example 6, wherein FK-506 and rapamycin reduced the amount of signal from the conjugate at concentrations greater than 0.1 nM and ascomycin reduced the signal at concentrations greater than 1 nM.

Example 12

Detection Of FK-506 In Whole Blood

When performing an assay for determining immunosuppressive agents from a whole blood test sample employing the immunosuppressant assay reagent of the present invention, it may be necessary to separate the immunosuppressive agent from other components in the whole blood sample which may cause an interference in the assay. In the present example, a whole blood sample from a mouse was treated with acetonitrile, an organic solvent, to separate ascomycin from substances which may interfere in an assay.

CD-1 mice were dosed with 5 mg/kg ascomycin orally and by intravenous injection. At intervals after dosing, the mice were euthanized with carbon dioxide and exsanguinated by cardiac puncture. Plasma was separated from red cells by centrifugation. Ascomycin was recovered from blood and plasma samples by treatment with water and acetonitrile. Samples were mixed with an equal volume of water for 30 seconds and acetonitrile was added to achieve an acetonitrile:water:sample ratio of 80:10:10 (v/v). After 16 hours at -20°C, the samples were centrifuged at 1000 X g for 10 minutes and the supernatants removed. The supernatants were dried *in vacuo* at ambient temperature, and the dried samples were then dissolved in the PBS/BSA/Tween 20 buffer and the amount of ascomycin was determined according to the assay method described in Example 11. A standard curve was prepared with ascomycin concentrations in the range of 0.1 to 10 nM. The ascomycin content in the blood and plasma samples was determined by comparing the amount of conjugate signal produced when the sample was included in the assay to the amount of conjugate signal produced when known amounts of ascomycin were included in the assay (Figures 7A and 7B).

Example 13

Binding Of Calcineurin To FKBP-CKS Immunosuppressant Assay Reagent

An binding assay was performed employing calcineurin and the FKBP-CKS immunosuppressant assay reagent as described in Example 1. The FKBP-CKS immunosuppressant assay reagent was dissolved at a concentration of 10-35 µg/ml in 20 mM sodium phosphate buffer, pH 7.4, 100 µl of the immunoassay reagent solution was added to the wells of a Immuno Plate MaxiSorp™ (Nunc, Naperville, IL), and incubated at ambient temperature for 2 hours in order to immobilize the immunosuppressant assay

reagent to the walls of the wells. To reduce the nonspecific binding of the ligand, 100 µl of a solution of phosphate buffered saline (PBS), pH 7.4, containing 2 % bovine serum albumin (BSA) and 0.2 % Tween 20 was added to the wells and incubated for 30 minutes at ambient temperature. The wells were emptied and rinsed with 0.2 % Tween 20 in PBS, and 50 µl of a solution containing FK-506 in 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 6 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM dithiothreitol, 0.2 % Tween 20, and 0.1 mg BSA per ml (Binding Buffer), or Binding Buffer alone, was added to the wells. An equal volume of a solution containing bovine brain calcineurin (Sigma® Chemical Co., St. Louis, MO) at 20 nM in Binding Buffer were added to the wells and incubated for 3 hours at ambient temperature. The wells were emptied and rinsed with 0.2 % Tween 20 in PBS, and a solution containing 20 mM para-nitrophenyl phosphate (substrate for calcineurin which produces para-nitrophenol, a yellow product).and 20 nM calmodulin (Sigma® Chemical Co., St. Louis, MO) was added to the wells and incubated for 16 hours at 37°C. The temporal change in 405 nm absorbance was recorded to determine the rate at which para-nitrophenol was formed.

The temporal change in 405 nm absorbance was related to the amount of calcineurin bound to the immobilized immunosuppressant assay reagent in the wells. As shown in Figure 8, some macrolide immunosuppressants such as FK-506 enhance the binding of calcineurin to the FKBP-CKS immunusuppressant assay reagent and; therefore, increase the amount of para-nitrophenol product produced therefrom. However, other macrolide immunosuppressants, like rapamycin, are unable to enhance calcineurin binding to the FKBP-CKS and can not be directly measured with this method.

Figure 9 demonstrates the ability of FK-506 and a structurally related analog, ascomycin, to increase in a concentration dependent manner the amount of signal produced when the macrolides are included in the assay of this Example 13. In this case, FK-506 and ascomycin are detected when they are present in the assay at concentrations greater than 1 nM. Because the level of signal is related to the concentration of the macrolide compound, standard curves can be created to relate the known concentration of a purified FK-506 to the amount of signal produced and used to estimate the unknown amount of the FK-506 in a sample.

Example 14

Binding Assay For FKBP

The FKBP-CKS immunosuppressant assay reagent as described in Example 1 was immobilized to the well walls of a polyvinylchloride microtiter plate by incubating a 10 ug/mL solution thereof, in 20 mM tris, 0.9% NaCl, pH7.2(TBS), in the wells for 30 minutes . The wells were then blocked with 1% BSA, 0.2% Tween 20 in BSA.

In order to demonstrate that immobilized FKBP is useful in an assay format to determine the binding activity of FK-506 or to quantitate the activity of FKBP, 0.5 ug/ml of an ascomycin-alkaline phosphatase conjugate as described in Example 2d was added to each of the microtiter plate wells in the presence of increasing concentrations of FKBP in 1% BSA and 0.2% tween 20 in TBS and incubated for 45 minutes at 37°C. The unbound conjugate was washed, and 100 ul of an alkaline phosphatase substrate solution (10 mg of para-nitrophenyl phosphate in 12.5 ml of diethanolamine buffer; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) for the conjugate was added.and incubated for 60 minutes at room temperature. Absorbance of the reaction between the conjugate and the substrate was measured at 405 nm and, as shown in Figure 10, a standard curve was generated by the soluble FKPB which can be employed to determine the binding activity of FK-506, or to quantitate the activity of FKBP, in the range of between about 1 ug/ml and about 40 ug/ml thereof.

Example 15

Immobilization of FKBP-CKS Immunosuppressant Assay Reagent With Anti-CKS Antibody

(a) Mouse monoclonal anti-CKS antibodies and goat polyclonal anti-CKS were each immobilized to the well walls of two polyvinyl chloride "U" bottom microtiter plates (Dynatech Laboratories Inc., Chantilly, VA), respectively, by adding 100µl of a 10 ug/ml solution of the antibody dissolved in Elisa Coating Buffer (ECB; per liter: 1.6 g $Na_2CO_3$, 2.9 g $NaHCO_3$, and 0.2 g $NaN_3$, pH 9.5-9.7). The microtiter wells were blocked by incubation for 15 minutes with a solution containing 10 mM Tris, 0.9 % NaCl, 0.05 % $NaN_3$, 1 % BSA, and 0.2 % Tween 20, pH 7.4 (TBS-BSA-T;150 µl per well). The FKBP-CKS immunosuppressant assay reagent as described in Example 1 was added to the wells of the microtiter plates at a concentration of 50 ng/ml in TBS-BSA-T (100

µl/well) and incubated one hour at room temperature. The wells were emptied and rinsed with plate washing buffer (0.9 % NaCl, 0.1 % Azide, and 0.1 % Tween 20). An ascomycin-alkaline phosphatase conjugate in TBS-BSA-T was added to the wells (100 ul/well) of each microtiter plate at a final concentration of 2 ug/ml in the presence of increasing concentrations of FK-506 (Figure 11) and incubated 1 hour at room temperature. The wells were emptied and rinsed with plate washing buffer and 100 ul of an alkaline phosphatase substrate solution (10 mg of para-nitrophenyl phosphate in 12.5 ml of diethanolamine buffer; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was added to each well. The amount of enzyme activity of the conjugate bound to the immobilized FKBP-CKS immunosuppressant assay reagent was quantitated by calculating the difference in the 405 nm absorbance of the wells at 5 minutes and 60 minutes. As shown in Figure 11 (superimposition of the curves indicates the close control of bound activity achievable with anti-CKS antibody immobilization), immobilization of the FKBP-CKS immunosuppressant assay reagent through monoclonal and polyclonal antibodies demonstrated that specific binding of the conjugate was more than 90% inhibitable by free FK-506 at 10 ng/mL

(b) In order to demonstrate that immobilized anti-CKS antibodies can be employed to separate free FK-506 from FK-506 bound to FKBP, the FKBP-CKS immunosuppressant assay reagent was reacted with an ascomycin-alkaline phosphatase conjugate in the presence of 1-10 ng/ml of free FK-506. The various reactions mixtures were then added to the wells of microtiter plates as described in paragraph (a) of this Example 15. As shown by Figure 12, the dose response curves obtained from the reaction mixtures in both of the microtiter plates demonstrate that separation of FKBP-CKS immunosuppressant assay reagent from free FK-506 by reaction with the CKS antibodies is useful for the determination of the presence of free FK-506.

It will be apparent that many modifications and variations of the present invention as herein set forth are possible without departing from the spirit and scope hereof, and that, accordingly, such limitations are imposed only as indicated by the appended claims.

We Claim:

1. A recombinant fusion protein for binding immunophilin ligands, said recombinant fusion protein comprising an immunosuppressant binding protein fused to a heterologous protein, said immunosuppressant binding protein being capable of binding an immunophilin ligand.

2. The fusion protein of claim 1 wherein said immunophilin ligand is selected from the group consisting of immunosuppressive agents and non-immunosuppressive agent capable of interacting with immunosuppressant binding proteins, and biologically-active metabolites, derivatives and analogs of said immunosuppressive agents and said non-immunosuppressive agents.

3. The fusion protein of claim 2 wherein said immunosuppressive agents are selected from the group consisting of FK-506, rapamycin, cyclosporin and ascomycin, and analogs and derivatives thereof.

4. The fusion protein of claim 1 wherein said immunosuppressant binding protein is selected from the group consisting of rapamycin binding protein, FK-506 binding protein, ascomycin binding protein and cyclosporin binding protein.

5. The fusion protein of claim 1 wherein said heterologous protein is selected from the group consisting of CTP:CMP-3-deoxy-D-*manno*-octulosonate cytidylyl transferase, glutathione S-transferase and the Fc portion of immunoglobulin molecules.

6. The fusion protein of claim 1 wherein said heterologous protein is CTP:CMP-3-deoxy-D-*manno*-octulosonate cytidylyl transferase.

7. A method for evaluating an immunophilin ligand or immunophilin in a test sample, said method comprising the steps of:

(a) contacting said test sample with (i) an immunosuppressant assay reagent comprising a recombinant fusion protein of an immunosuppressant binding protein and a heterologous protein, wherein said immunosuppressant binding protein is capable of binding an immunophilin ligand, and (ii) a detectable reagent capable of providing a detectable signal; and

(b) measuring the amount of said detectable reagent from step (a) which either has or has not participated in a binding reaction with said immunosuppressant assay reagent as a function of said immunophilin ligand or said immunophilin in said test sample.

8. The method of claim 7 wherein said detectable reagent comprises an immunophilin ligand or analog thereof labeled with a detectable moiety.

9. The method of claim 7 wherein said detectable reagent comprises an immunophilin labeled with a detectable moiety.

10. The method of claim 7 wherein said detectable reagent comprises an immunoreactant labeled with a detectable moiety.

11. The method of claim 7 wherein said detectable reagent is a calcium /calmodulin activated phosphatase for evaluating an immunophilin ligand in said test sample.

12. The method of claim 7 wherein said immunophilin ligand is selected from the group consisting of immunosuppressive agents and non-immunosuppressive agents capable of interacting with immunosuppressant binding proteins, and biologically-active metabolites, derivatives and analogs of said immunosuppressive agents and said non-immunosuppressive agents.

13. The method of claim 12 wherein said immunosuppresssive agents are selected from the group consisting of FK-506, rapamycin, cyclosporin and ascomycin, and analogs and thereof.

14. The method of claim 7 wherein said immunosuppressant binding protein is selected from the group consisting of FK-506 binding protein, rapamycin binding protein, ascomycin binding protein and cyclosporin binding protein.

15. The method of claim 7 wherein said heterologous protein is selected from the group consisting of CTP:CMP-3-deoxy-D-*manno*-octulosonate cytidylyl transferase, glutathione S-transferase and the Fc portion of immunoglobulin molecules.

16. The method of claim 7 wherein said heterologous protein is CTP:CMP-3-deoxy-D-*manno*-octulosonate cytidylyl transferase.

17. A heterogeneous assay method for evaluating an immunophilin ligand in a test sample, said method comprising the steps of:

(a) contacting said test sample with (i) an immunosuppressant assay reagent comprising a recombinant fusion protein of an immunosuppressant binding protein and a heterologous protein immobilized to a solid phase material through said heterologous protein, wherein said immunosuppressant binding protein is capable of binding an immunophilin ligand, and (ii) a detectable reagent capable of providing a detectable signal; and

(b) measuring the amount of said detectable reagent from step (a) which either has or has not participated in a binding reaction with said immunosuppressant assay reagent as a function of said immunophilin ligand in said test sample.

18. The method of claim 17 wherein said immunosuppressant assay reagent is immobilized to said solid phase material by a covalent bond.

19. The method of claim 17 wherein said immunosuppressant assay reagent is immobilized to said solid phase material by adsorption.

20. The method of claim 17 wherein said immunosuppressant assay reagent is immobilized to said solid phase material with an immunoreactant for said heterologous protein immobilized to said solid phase material whereby said heterologous protein of said immunosuppressant assay reagent is bound to said immobilized immunoreactant.

21. The method of claim 17 wherein said immunosuppressant assay reagent further comprises a first binding member of a specific binding member pair coupled to said heterologous protein, wherein said immunosuppressant assay reagent is immobilized to said solid phase material with a second binding member of said specific binding member pair immobilized to said solid phase material whereby said first binding member is bound to said immobilized second binding member.

22. The method of claim 17 wherein said detectable reagent comprises an immunophilin ligand or analog thereof labeled with a detectable moiety.

23. The method of claim 17 wherein said detectable reagent comprises an immunophilin labeled with a detectable moiety.

24. The method of claim 17 wherein said detectable reagent comprises an immunoreactant labeled with a detectable moiety.

25. The method of claim 17 wherein said detectable reagent is a calcium/calmodulin activated phosphatase.

26. The method of claim 17 wherein said immunophilin ligand is selected from the group consisting of immunosuppressive agents and non-immunosuppressive agents capable of interacting with immunosuppressant binding proteins, and biologically-active metabolites, derivatives and analogs of said immunosuppressive agents and said non-immunosuppressive agents.

27. The method of claim 17 wherein said immunosuppresssive agents are selected from the group consisting of FK-506, rapamycin, cyclosporin and ascomycin, and analogs and derivatives thereof.

28. The method of claim 17 wherein said immunosuppressant binding protein is selected from the group consisting of FK-506 binding protein, rapamycin binding protein, ascomycin binding protein and cyclosporin binding protein.

29. The method of claim 17 wherein said heterologous protein is selected from the group consisting of CTP:CMP-3-deoxy-D-*manno*-octulosonate cytidylyl transferase, glutathione S-transferase and the Fc portion of immunoglobulin molecules.

30. The method of claim 17 wherein said heterologous protein is CTP:CMP-3-deoxy-D-*manno*-octulosonate cytidylyl transferase.

31 A test kit for evaluating an immunophilin ligand or immunophilin in a test sample, said test kit comprising:

(a) an immunosuppressant assay reagent comprising a recombinant fusion protein of an immunosuppressant binding protein and a heterologous protein, wherein said immunosuppressant binding protein is capable of binding an immunophilin ligand, and

(b) a detectable reagent capable of providing a detectable signal.

32. The test kit of claim 31 wherein said detectable reagent comprises an immunophilin ligand or analog thereof labeled with a detectable moiety.

33. The test kit of claim 31 wherein said detectable reagent comprises an immunophilin labeled with a detectable moiety.

34. The test kit of claim 31 wherein said detectable reagent comprises an immunoreactant labeled with a detectable moiety.

35. The test kit of claim 31 wherein said detectable reagent is a calcium/calmodulin activated phosphatase for evaluating an immunophilin ligand in said test sample.

36. The test kit of claim 31 wherein said immunophilin ligand is selected from the group consisting of immunosuppressive agents and non-immunosuppressive agents capable of interacting with immunosuppressant binding proteins, and biologically-active metabolites, derivatives and analogs of said immunosuppressive agents and said non-immunosuppressive agents.

37. The test kit of claim 31 wherein said immunosuppresssive agents are selected from the group consisting of FK-506, rapamycin, cyclosporin and ascomycin, and analogs and derivatives thereof.

38. The test kit of claim 31 wherein said immunosuppressant binding protein is selected from the group consisting of FK-506 binding protein, rapamycin binding protein, ascomycin binding protein and cyclosporin binding protein.

39. The test kit of claim 31 wherein said heterologous protein is selected from the group consisting of CTP:CMP-3-deoxy-D-*manno*-octulosonate

cytidylyl transferase, glutathione S-transferase and the Fc portion of immunoglobulin molecules.

40. The test kit of claim 31 wherein said heterologous protein is CTP:CMP-3-deoxy-D-*manno*-octulosonate cytidylyl transferase.

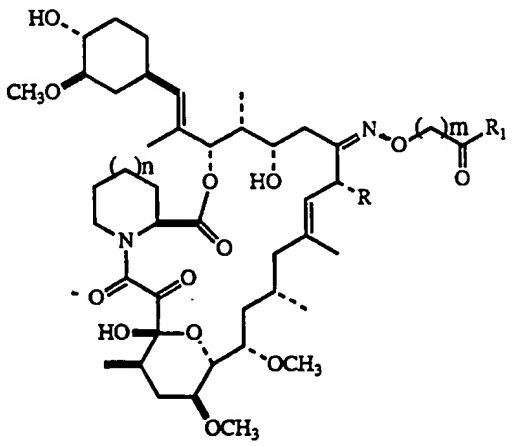
41. The test kit of claim 31 wherein said immunosuppressant assay reagent is immobilized to said solid phase material by a covalent bond.

42. The test kit of claim 31 wherein said immunosuppressant assay reagent is immobilized to said solid phase material by adsorption.

43. The test kit of claim 31 wherein said immunosuppressant assay reagent is immobilized to said solid phase material with an immunoreactant for said heterologous protein immobilized to said solid phase material whereby said heterologous protein of said immunosuppressant assay reagent is bound to said immobilized immunoreactant.

44. The test kit of claim 31 wherein said immunosuppressant assay reagent further comprises a first binding member of a specific binding member pair coupled to said heterologous protein, wherein said immunosuppressant assay reagent is immobilized to said solid phase material with a second binding member of said specific binding member pair immobilized to said solid phase material whereby said first binding member is bound to said immobilized second binding member.

45. A compound of the formula:



wherein n is the integer 0 or 1;

m is an integer from 0-6;

R is selected from the group consisting of methyl, ethyl, propyl and allyl; and

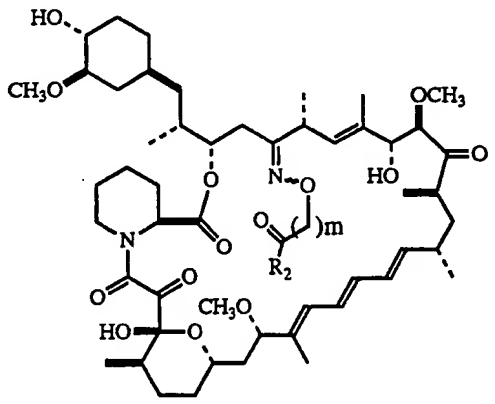
$R_1$ is OH or NH-X, wherein NH-X is a macromolecule or a detectable moiety.

46. The compound of claim 45 wherein NH-X is a macromolecule selected from the group consisting of bovine serum albumin, keyhole limpet hemocyanin and thyroglobulin.

47. The compound of claim 45 wherein NH-X is a detectable moiety selected from the group consisting of enzymatically active groups, chromophores, fluorescent molecules, chemiluminescent molecules, phosphorescent molecules and luminescent molecules.

48. The compound of claim 45 wherein n=1, m=1, R=ethyl and $R_1$ is NH-X where NH-X is alkaline phosphatase.

49. A compound of the formula:




wherein m is an integer from 0-6; and

$R_2$ is OH or NH-Y, wherein NH-Y is a macromolecule or a detectable moiety.

50. The compound of claim 49 wherein NH-Y is a macromolecule selected from the group consisting of bovine serum albumin, keyhole limpet hemocyanin and thyroglobulin.

51. The compound of claim 49 wherein NH-Y is a detectable moiety selected from the group consisting of enzymatically active groups, chromophores, fluorescent molecules, chemiluminescent molecules, phosphorescent molecules and luminescent molecules.

52. The compound of claim 49 wherein m=1 and $R_2$ is NH-Y where NH-Y is alkaline phosphatase.

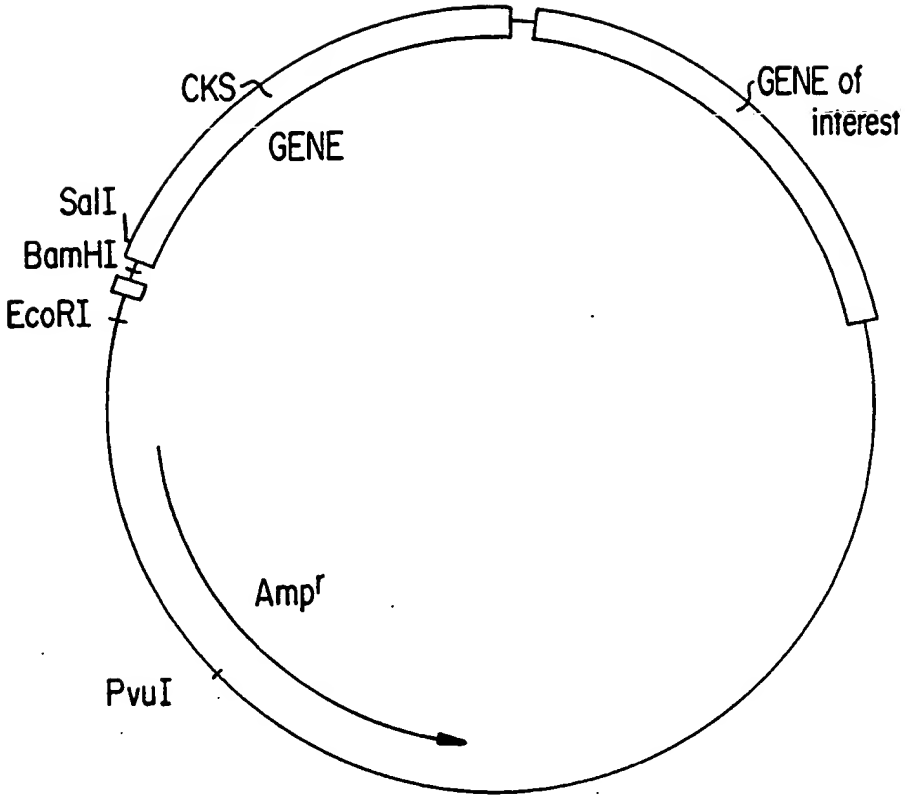



FIG. 1

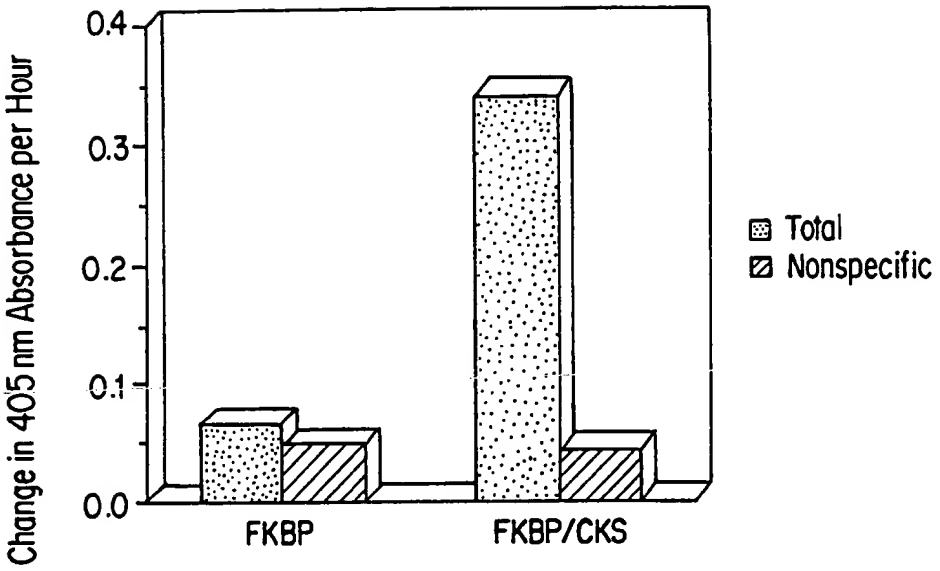

Change in 405 nm Absorbance per Hour
0.4
0.3
0.2
0.1
0.0
FKBP
FKBP/CKS
Total
Nonspecific

FIG. 2

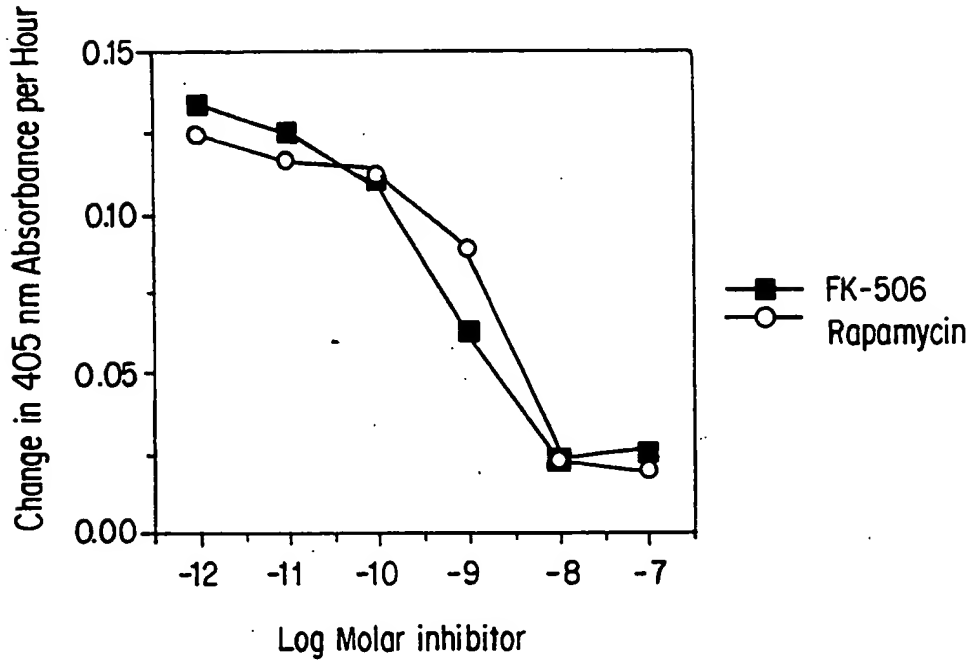

Change in 405 nm Absorbance per Hour
0.15
0.10
0.05
0.00
-12
-11
-10
-9
-8
-7
Log Molar inhibitor
FK-506
Rapamycin

FIG. 3

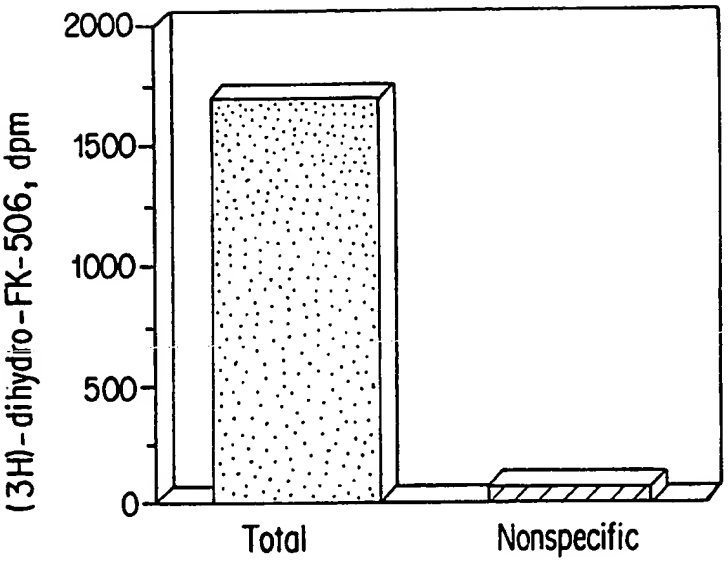

2000
1500
1000
500
0
(3H)-dihydro-FK-506, dpm
Total
Nonspecific

FIG. 4

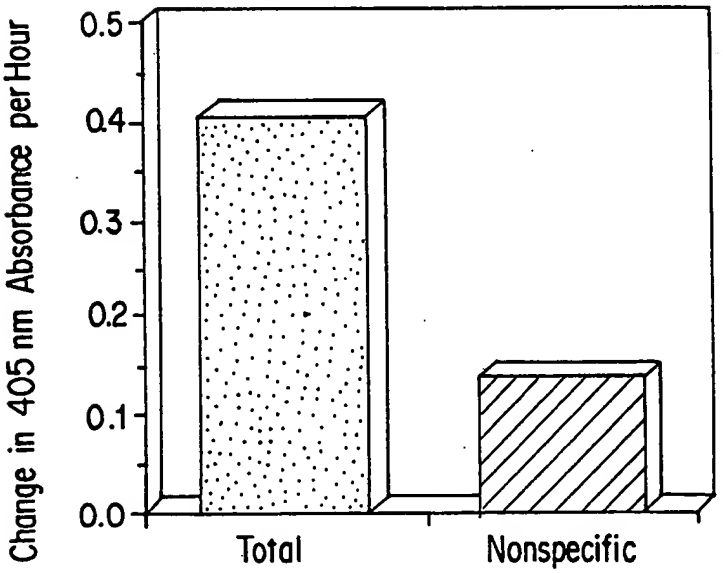

0.5
0.4
0.3
0.2
0.1
0.0
Change in 405 nm Absorbance per Hour
Total
Nonspecific

FIG. 5

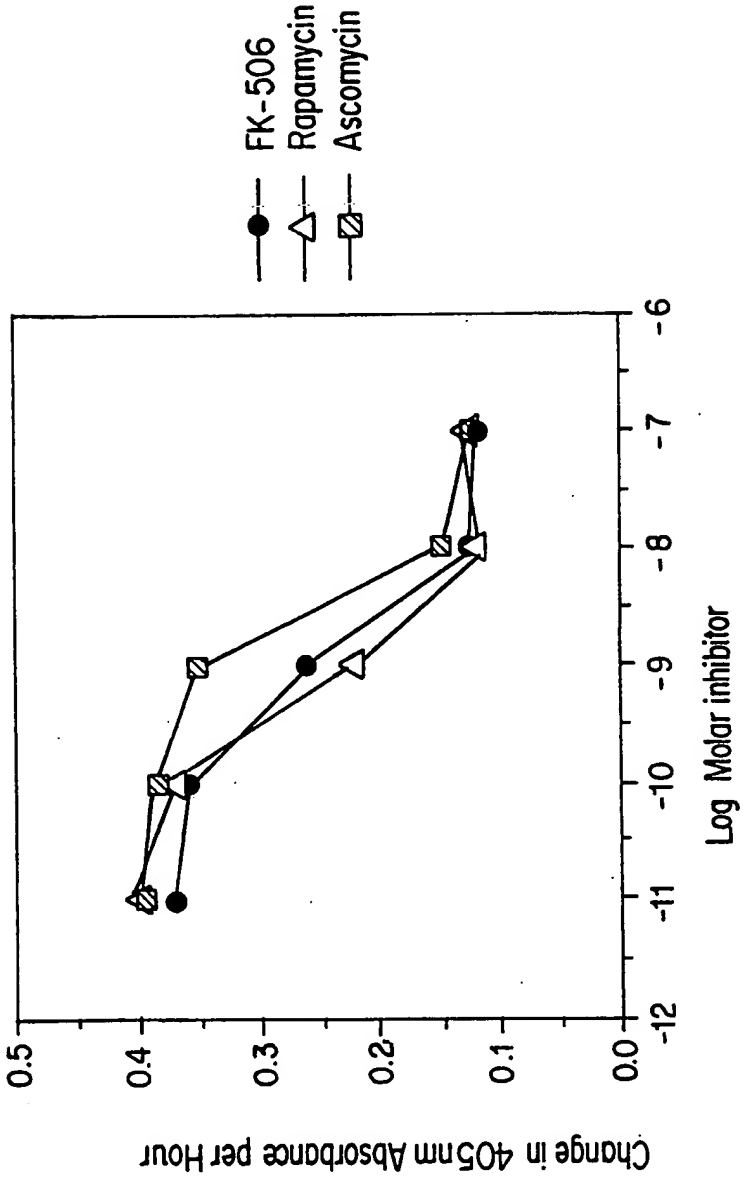

FK-506
Rapamycin
Ascomycin
Change in 405nm Absorbance per Hour
0.5
0.4
0.3
0.2
0.1
0.0
-12
-11
-10
-9
-8
-7
-6
Log Molar inhibitor

FIG. 6

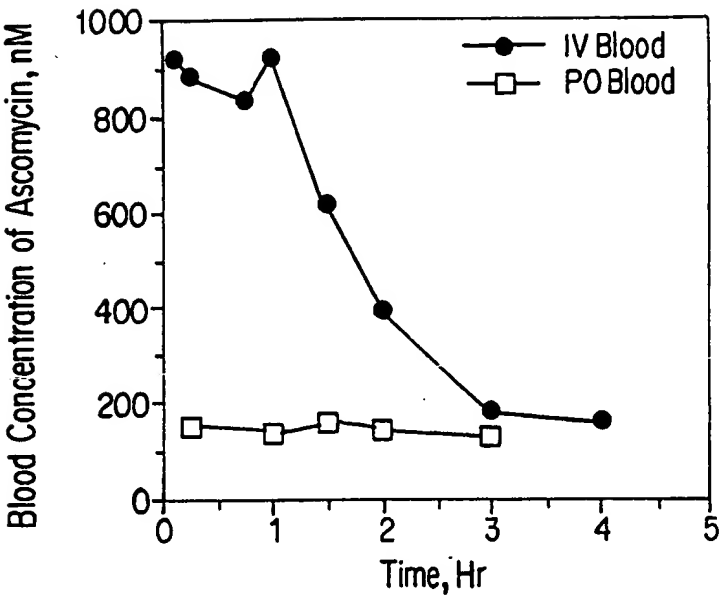

Blood Concentration of Ascomycin, nM
1000
800
600
400
200
0
0
1
2
3
4
5
Time, Hr
IV Blood
PO Blood

FIG. 7A

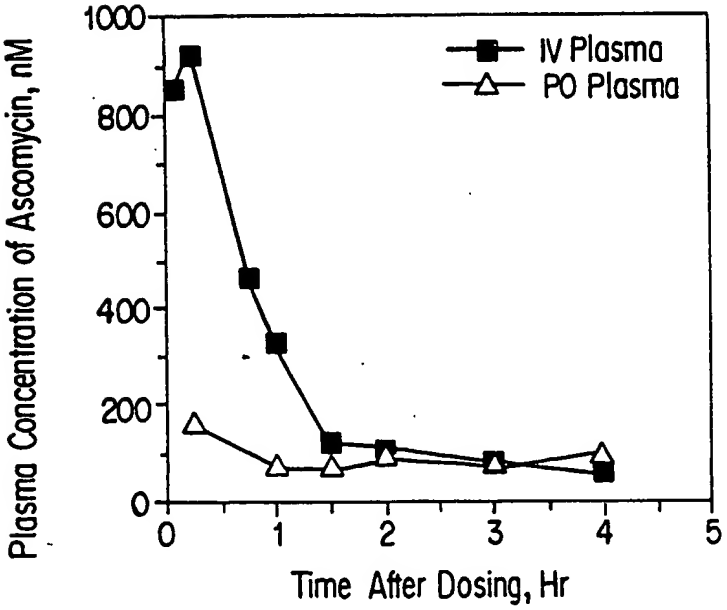

Plasma Concentration of Ascomycin, nM
1000
800
600
400
200
0
0
1
2
3
4
5
Time After Dosing, Hr
IV Plasma
PO Plasma

FIG. 7B

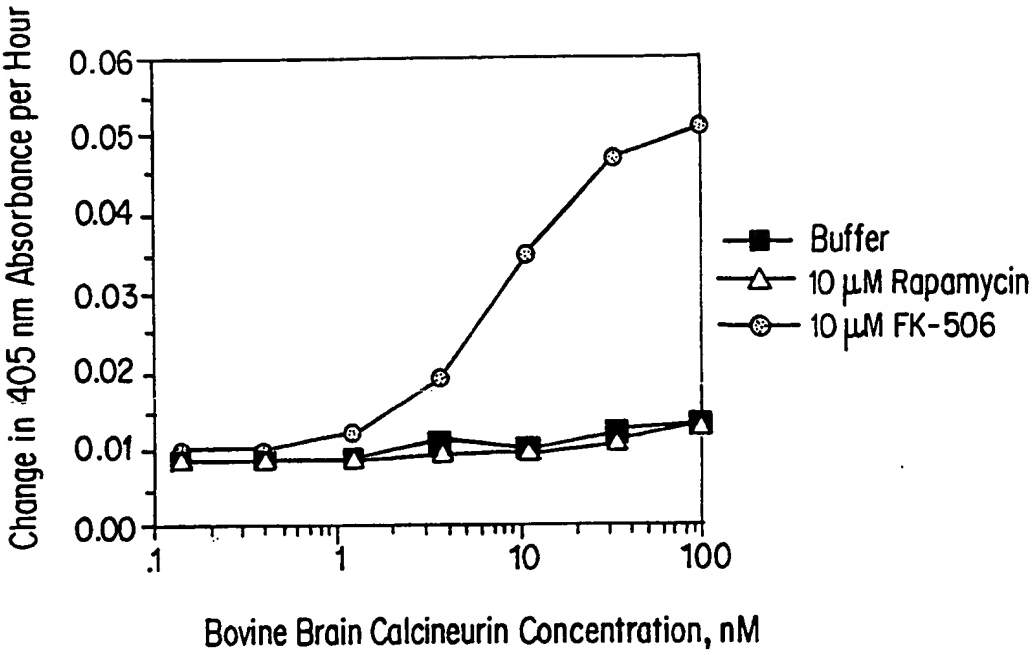

Change in 405 nm Absorbance per Hour
0.06
0.05
0.04
0.03
0.02
0.01
0.00
.1
1
10
100
Bovine Brain Calcineurin Concentration, nM
Buffer
10 µM Rapamycin
10 µM FK-506

FIG. 8

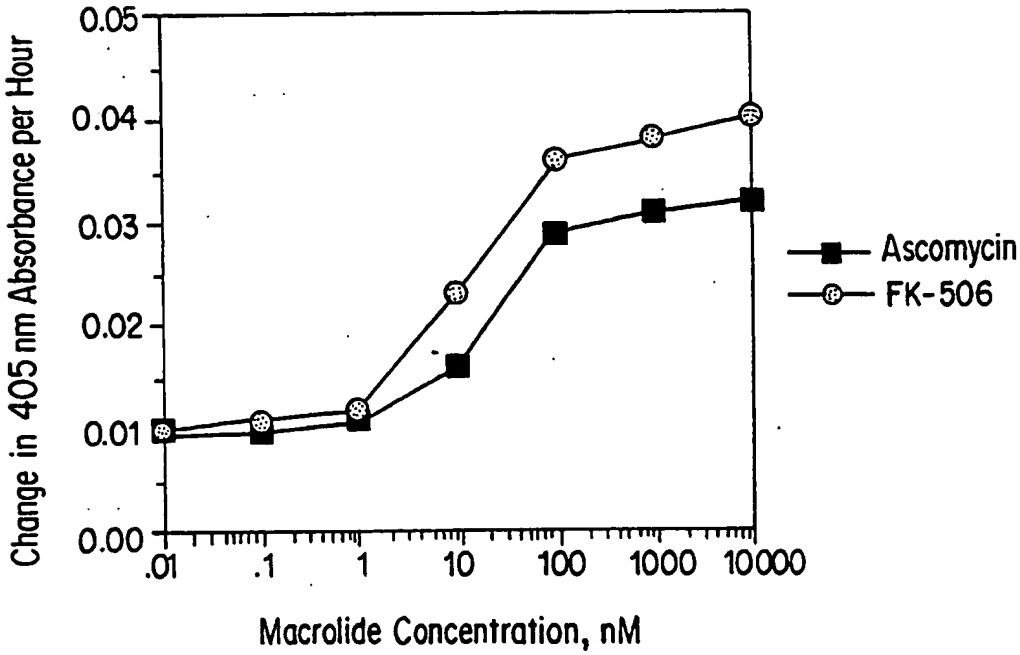

Change in 405 nm Absorbance per Hour
0.05
0.04
0.03
0.02
0.01
0.00
.01
.1
1
10
100
1000
10000
Macrolide Concentration, nM
Ascomycin
FK-506

FIG. 9

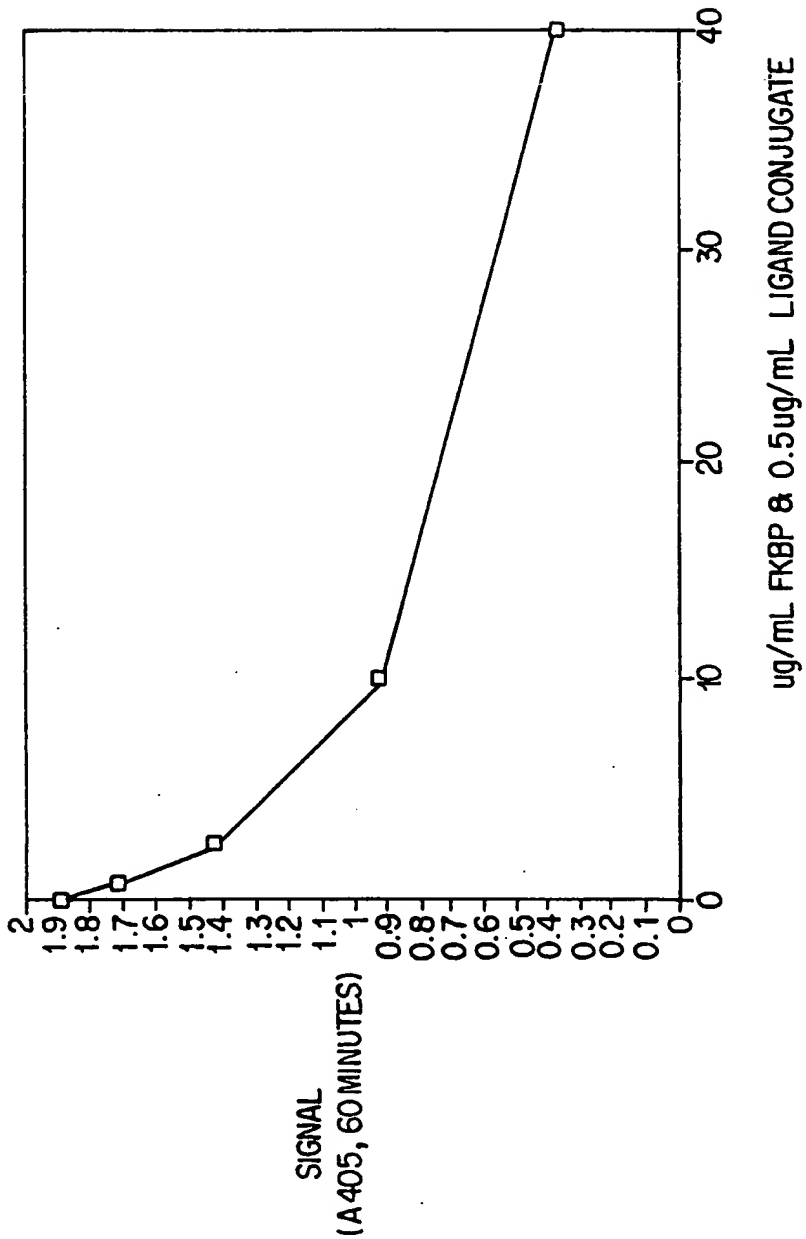

SIGNAL
(A 405, 60 MINUTES)
2
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
ug/mL FKBP & 0.5ug/mL LIGAND CONJUGATE

FIG. 10

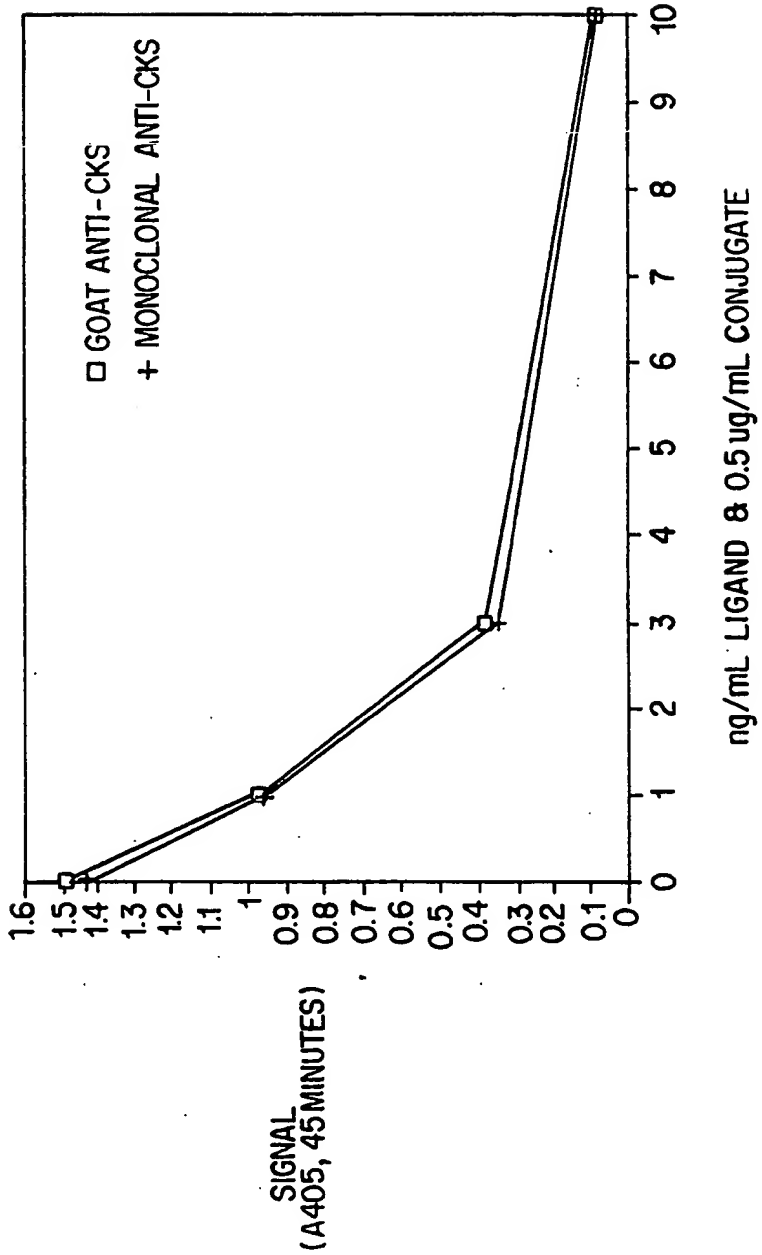

□ GOAT ANTI-CKS
+ MONOCLONAL ANTI-CKS
SIGNAL
(A405, 45 MINUTES)
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 1 2 3 4 5 6 7 8 9 10
ng/mL LIGAND & 0.5 ug/mL CONJUGATE

FIG.11

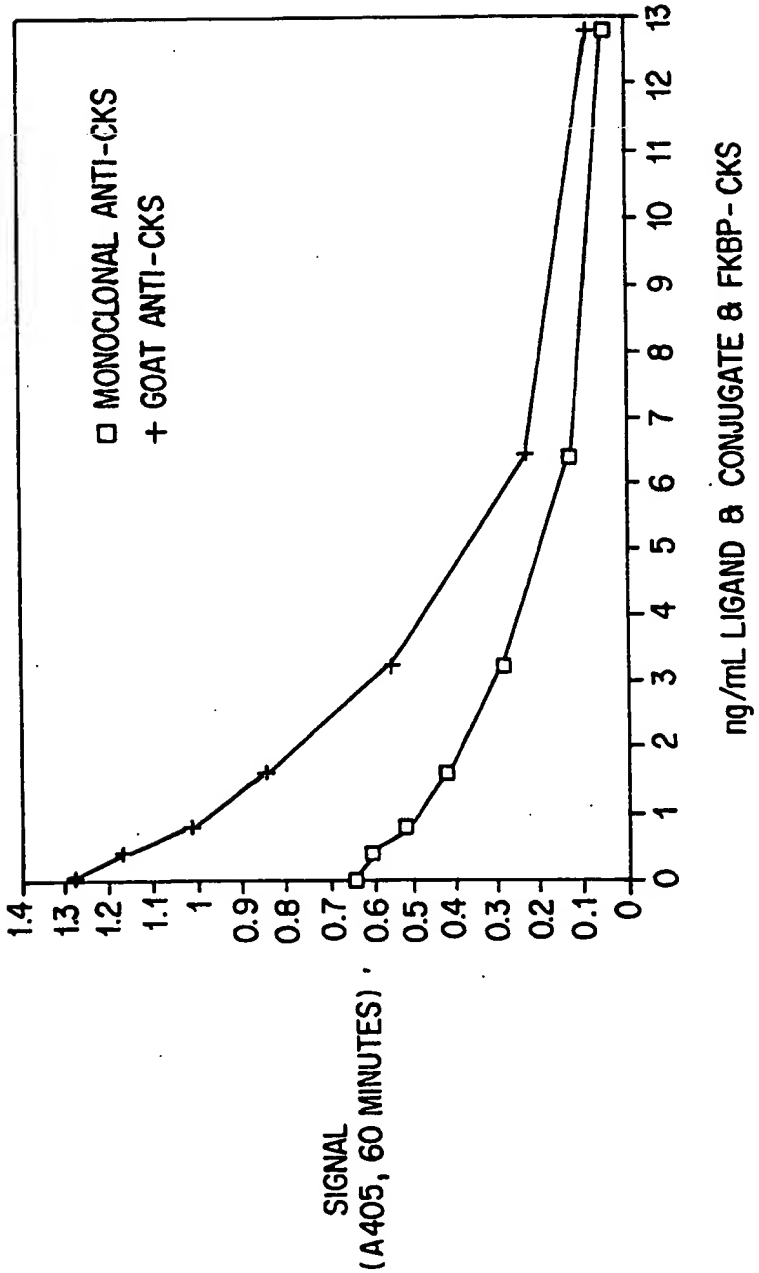
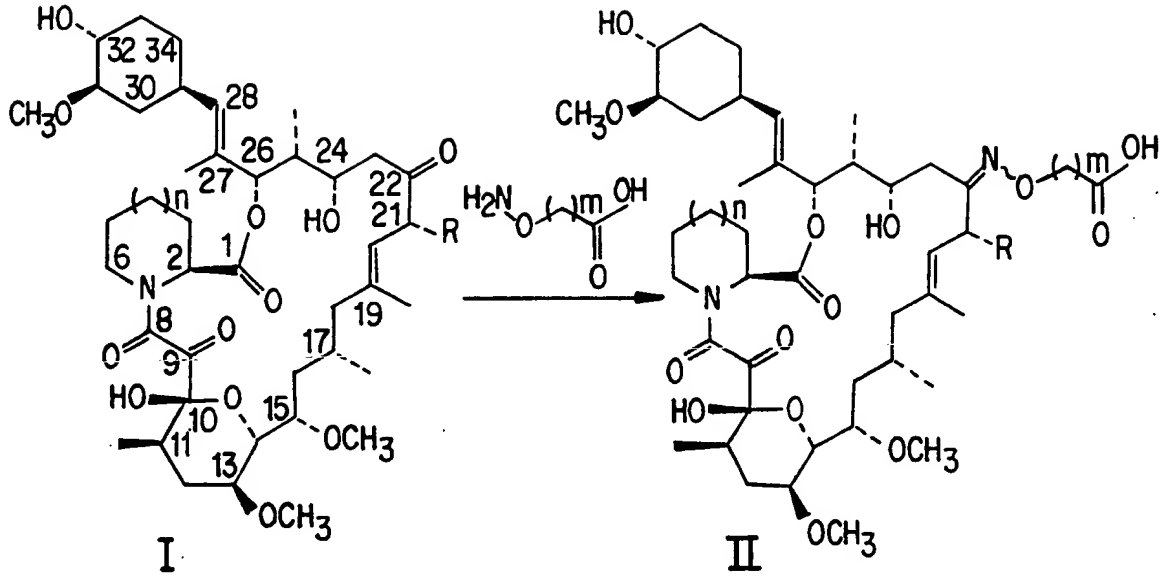

□ MONOCLONAL ANTI-CKS
+ GOAT ANTI-CKS
SIGNAL
(A405, 60 MINUTES)
ng/mL LIGAND & CONJUGATE & FKBP-CKS
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
1.1
1.2
1.3
1.4
0
1
2
3
4
5
6
7
8
9
10
11
12
13

FIG. 12




FK-506: n=1, R=allyl
Ascomycin(FR-900520): n=1, R=ethyl
FK-523: n=1, R=methyl
FK-525: n=0, R=allyl
Dihydro-FK-506: n=1, R=propyl

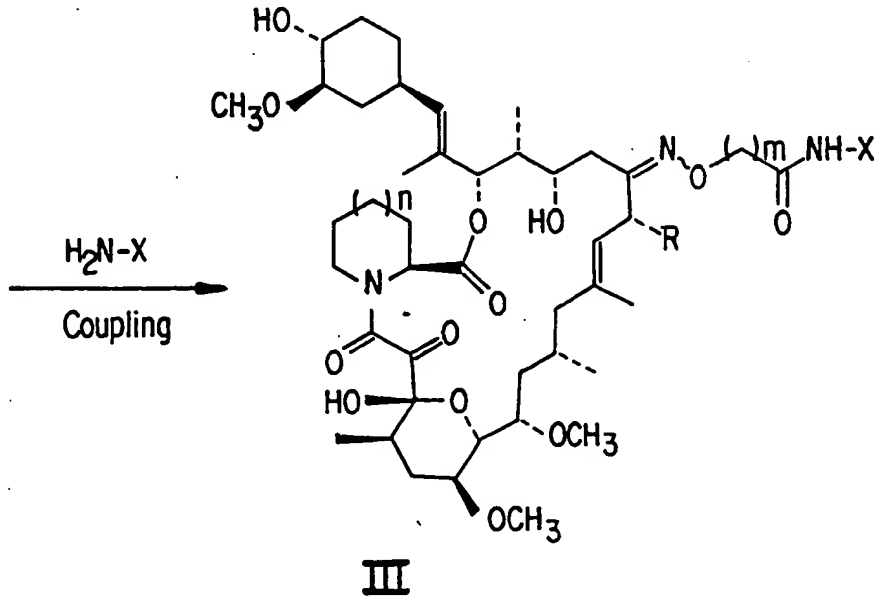



FIG. 13

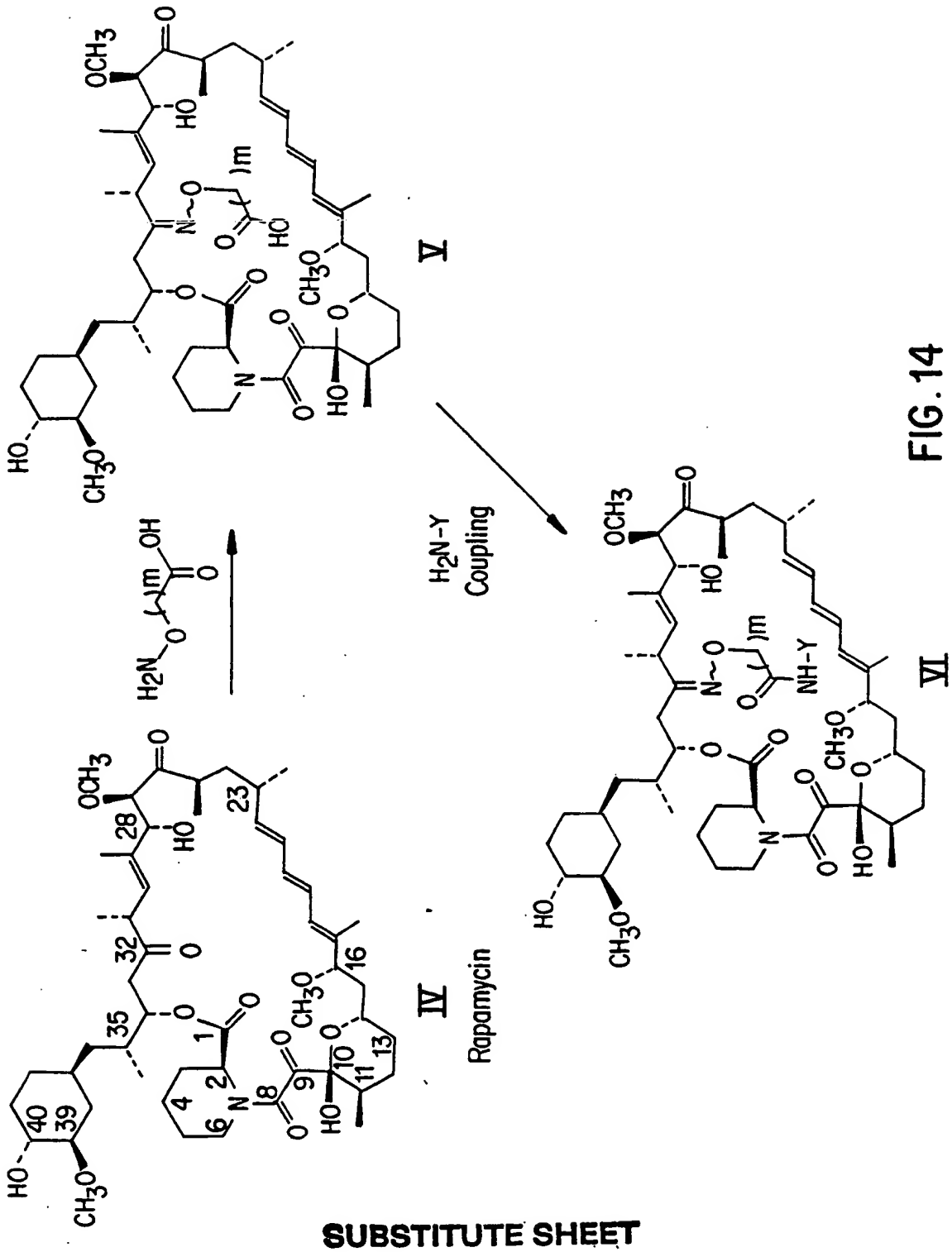



FIG. 14

# INTERNATIONAL SEARCH REPORT

Inte.national application No.
PCT/US93/05197

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :435/7.1; 530/350; 546/26

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1; 530/350; 546/26

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| E | US, A, 5,196,352 (SIEKIERKA ET AL.) 23 MARCH 1993, see entire document. | 1-44 |
| & | US, A, 5,124,255 (BOLLING ET AL.) 23 JUNE 1993, see entire document. | 1-44 |
| Y | US, A, 5,109,112 (SIEKIERKA ET AL.) 28 APRIL 1992, see entire document. | 1-44 |
| Y | US, A, 5,047,512 (HANDSCHUMACHER ET AL.) 10 SEPTEMBER 1991, see entire document. | 1-44 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be part of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

Date of the actual completion of the international search

24 August 1993

Date of mailing of the international search report

SEP 07 1993

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

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Form PCT/ISA/210 (second sheet)(July 1992)*

## INTERNATIONAL SEARCH REPORT

Inte..ational application No.
PCT/US93/05197

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | SCIENCE, Volume 226, issued 02 November 1984, R.E. Handschumacher et al., "Cyclophilin: A Specific Cytosolic Binding Protein for Cyclosporin A", pages 544-547, see entire document. | 1-44 |
| Y | THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 261, number 34, issued 05 December 1986, R.C. Goldman et al., "Primary Structure of CTP:CMP-3-deoxy-D-manno-octulosonate Cytidylyltransferase (CMP-KDO Synthetase) from Escherichia coli", pages 15831-15835, see entire document. | 1-44 |
| Y | TRANSPLANTATION PROCEEDINGS, Volume 22, number 3, issued June 1990, M.I. Lorber et al., "Cyclophilin Binding: A Receptor-Mediated Approach to Monitoring Cyclosporine Immunosuppressive Activity Following Organ Transplantation", pages 1240-1244, see entire document. | 1-44 |
| Y | N.R. ROSE et al., "MANUAL OF CLINICAL IMMUNOLOGY", published 1976 by AMERICAN SOCIETY FOR MICROBIOLOGY (WASHINGTON, D.C.), see pages 506-512, see entire document. | 1-44 |
| Y | BIOTECHNIQUES, Volume 8, number 5, issued 1990, T.J. Bolling et al., "An Escherichia coli Expression Vector for High-Level Production of Heterologous Proteins in Fusion with CMP-KDO Synthetase", pages 488-492, see entire document. | 1-44 |
| Y | CELL, Volume 66, issued 23 August 1991, J. Liu et al., "Calcineurin Is a Common Target of Cyclophilin-Cyclosporin A and FKBP-FK506 Complexes", pages 807-815, see entire document. | 1-44 |
| Y | EP, A, 0,293,892 (NIWA ET AL.) 07 DECEMBER 1988, see entire document, especially page 2. | 45-52 |
| Y | WO, A, 91/17439 (SOLDIN ET AL.) 14 NOVEMBER 1991, see entire document. | 1-52 |
| Y | EP, A, 0,184,162 (OKUHARA ET AL.) 11 JUNE 1986, see entire document, especially claims. | 1-52 |

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C07D 221/18, 221/22; C07K 3/00, 13/00, 15/00, 17/00; C12Q 1/00; G01N 33/53

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

STN-REGISTRY, CAS, CAPREVIEWS, MEDLINE, BIOSIS, EMBASE, DISSERTATION ABSTRACTS, LIFE SCIENCES COLLECTION, PASCAL, HEALTH PERIODICALS DATABAS, CANCERLIT, FEDERAL RESEARCH IN PROGRESS, DERWENT WORLD PATENTS INDEX, DERWENT BIOTECHNOLOGY ABS; APS

search terms: IMMUNOPHILI?, ASSAY?, IMMUNOASSAY?, FK (W)506, RAPAMYCIN, CYCLOSPORIN, ASCOMYCIN, BINDING (W) PROTEIN?, RECEPT!R?
RECOMBINANT, FUSION (W) PROTEIN?, CALCIUM, CALMODULIN, PHOSPHATAS?, IMMUNOSUPPRES?, LIGAND?, OCULOSONATE (W) CYTIDYLYL (W) TRANSFERASE?, CKS

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-44, drawn to the first appearing compositions (fusion proteins) and methods of using said compositions, classified in Class 530, subclass 350 and Class 435, subclass 7.1.

II. Claims 45-48 drawn to the second appearing composition comprising an immunosuppressive agent, classified in Class 536, subclass 26.

III. Claims 49-52, drawn to the third appearing composition comprising an immunosuppressive agent, classified in Class 536, subclass 26.

The inventions are distinct, one from the other because:

Invention I is drawn to a protein containing composition and methods of using said composition whereas Inventions II and III are drawn to heterocyclic organic compounds. The heterocylcic organic compounds are not required for the practice of the method of Group I.

The compositions of the three inventions are distinct, one from the other, because they comprise chemically distinct compounds each of which possesses separate utility. For example, the proteins of Group I may be used as detection agents in the assays of that Group, whereas the compounds of Groups II and III may be used as *in vivo* immunosuppressive agents.

The inventions of Groups II and III are distinct, one from the other because they are drawn to distinct chemical structures whose physical properties are not predictable one over the other and as such may have separate properties that would not be apparent from analysis of the either one.

The claimed invention further lacks unity of invention because the methods of the instant invention employ proteins and the compounds of Groups II and III are non-proteinacious. Therefore, the claimed inventions lack any special technical feature within the meaning of PCT rule 13.2, that would indicate that unity of invention is present.